Disruption of the splenic histological architecture associated with severe forms of visceral leishmaniasis

Desestruturação histoarquitetural do baço e susceptibilidade a formas graves da leishmaniose visceral

Washington Luis Conrado dos-Santos[1,2], Joselli Santos Silva[1] e Rita Elizabeth Moreira Mascarenhas[1,2]

ABSTRACT

Background: Severe forms of visceral leishmaniasis are associated with atrophy and histological disorganization of spleen compartments in both humans and dogs. **Methods:** In this paper, we review the changes observed in the spleens of dogs from an area endemic for visceral leishmaniasis. **Results:** Dogs with susceptibility markers for visceral leishmaniasis display disorganization of the splenic tissue more frequently than dogs without these markers (χ^2-test, P<0·01). This disorganization was more intense in the marginal zone and the lymphoid follicle than in the periarterial lymphoid sheath. In addition, the total number of T lymphocytes (Whitney, $P < 0.05$), B lymphocytes (Whitney, $P < 0.01$) and $S100^+$ dendritic cells (Whitney, $P < 0.01$) in the follicles and of B lymphocytes in the marginal zone (Whitney, $P < 0.01$) in animals with disorganized spleen architecture was lower than in animals with normal spleen histology. The architectural disorganization of the spleen is associated with lower expressions of LTα and CXCL13 than those observed in normal spleen. There is also a greater frequency of bacterial skin infections in animals with spleen cultures positive for *Leishmania* (47%) than in animals with negative cultures (21%). **Conclusion:** The architectural disorganization of the splenic tissue in dogs with susceptibility markers of visceral leishmaniasis is associated with aberrant expression of cytokines involved in the structuring of the follicles and marginal zone of the spleen. These splenic alterations may impair the leukocyte cooperation required for effective immunity against infection by *Leishmania* and other pathogens.

Key-words: Canine visceral leishmaniasis. Chemokines. Spleen histology. Lymphoid follicle. Marginal zone.

RESUMO

Introdução: Formas graves de leishmaniose visceral estão associadas com atrofia e desorganização dos compartimentos do baço em seres humanos e em cães. **Métodos:** Nesse artigo nós revimos as alterações observadas nos baços de cães de uma área endêmica de leishmaniose visceral. **Resultados:** Cães com marcadores de susceptibilidade a leishmaniose visceral apresentam mais frequentemente desorganização do tecido esplênico (χ^2-test, P<0·01) afetando mais intensamente a zona marginal e folículos linfoides a bainha periarteriolar de linfócitos. O número total de linfócitos T (Whitney, $P < 0.05$), linfócitos B (Whitney, $P < 0.01$) e células dendríticas $S100^+$ (Whitney, $P < 0.01$) nos folículos e de células B na zona marginal (Whitney, $P < 0.01$) dos animais com a arquitetura do baço desorganizada foi menor que nos animais com a histologia do baço normal. Essa desorganização arquitetural do baço está associada com menor expressão de LTα e CXCL13 que a observada em baços normais. Há maior frequência de infecções bacterianas de pele em animais com culturas esplênicas positivas para *Leishmania* (47%) que em animais com culturas negativas (21%). **Conclusão:** A desorganização arquitetural do tecido esplênico em cães é associada à expressão inadequada de citocinas envolvidas na estruturação dos folículos e da zona marginal do baço. Essas alterações do baço, presentes no curso da leishmaniose visceral pode prejudicar com a cooperação entre leucócitos na defesa contra a infecção por *Leishmania* e por outros patógenos.

Palavras-chaves: Leishmaniose visceral canina. Quimiocinas. Histologia do baço. Folículo linfóide. Zona marginal.

The spleen is an organ located in the left subcostal region, distal to the diaphragm, both in humans and in dogs. Although it can be removed in the course of treatment for certain diseases or traumatic injuries, the spleen exerts important functions in the body [1]. It is a secondary lymphoid organ that serves as the site for the late development of B lymphocytes [2] and for the priming and cooperation of T and B lymphocytes in producing antibody and mounting a cellular immune response against circulating pathogens [3,4]. The spleen is also the storage site for inflammatory monocytes, allowing the prompt release of large quantities of these cells to remote sites in response to acute injury [5]. The spleen eliminates old or damaged red blood cells in a process known as hemocateresis; it also recycles iron via a mechanism that is dependent on members of the natural-resistance-associated macrophage protein (NRAMP) family, limiting the availability of free iron, which is necessary for bacterial metabolism [6]. As a secondary lymphoid organ interposed in the blood stream, the spleen serves to monitor blood for circulating antigens and pathogens [1]. In dogs, in addition to these functions, the spleen works as a blood reservoir, allowing release of up to 81% of its blood volume in conditions of severe hemorrhage [7]. Hence, many of the spleen's functions converge towards innate and adaptive defense mechanisms against infection. The function of the spleen in defense against infection is evident in the fact that asplenic and hyposplenic individuals are more susceptible to infection and to bacterial, viral and fungal septicemia [1]. The cumulative risk of developing serious infection is almost 33% in the first 10 years after splenectomy performed for a variety of medical indications [1].

We decided to study the spleen in visceral leishmaniasis because (1) this organ is involved in all cases of the disease; (2) it is affected throughout the whole course of visceral leishmaniasis [8]; and (3) the main components involved in the immune response against the parasite are present in the spleen: infected cells and parasite antigens, antigen-presenting cells and T and B lymphocytes responding against *Leishmania* antigens. The importance of the spleen in clinical visceral leishmaniasis is highlighted by the fact that spleen regression is used as indicative of control of human infection in response to treatment [9]. Finally, we work with the hypothesis that severe forms of leishmaniasis generate a pattern of atrophy and disorganization of splenic tissue that may reproduce aspects of a hyposplenic or asplenic syndrome.

1. Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Fundação Oswaldo Cruz, Salvador, BA, Brazil. 2. Escola Bahiana de Medicina e Saúde Pública, Salvador, BA, Brazil.

Address to: Dr. Washington LC dos-Santos. Laboratório de Patologia e Bio-Intervenção/Centro de Pesquisas Gonçalo Moniz/FIOCRUZ. R. Waldemar Falcão 121, Candeal, 40296.710 Salvador, BA, Brazil.
e-mail: wluis@bahia.fiocruz.br

Since the initial autopsy reports on patients who died of visceral leishmaniasis, an association has emerged between severe forms of the disease and atrophy of the white pulp of the spleen [10]. Experimental models have revealed a more complex pattern of changes, varying from hyperplasia to atrophy according to the susceptibility of the host and the stage of disease [11-13]. In 1977, Veres and collaborators (1977) considered the possibility that the disruption of the architecture of lymphoid organs impairs leukocyte cooperation against *Leishmania* infection. These alterations in the architecture of splenic tissue have been associated with loss or change in the distribution of cell populations in the spleen, including plasma cells, replacement of lymphoid and red pulp areas, loss of marginal zone macrophages and follicular dendritic cells, and changes in the distribution pattern of B and dendritic cells [10-12, 14, 15]. Furthermore, similar disruption of splenic lymphoid tissue architecture has been described in viral infections [16]. Along with chemokines, TNF and IL-10 are the main cytokines implicated in the changes in spleen structure in experimental models of murine visceral leishmaniasis [14, 17].

A few years ago, we examined the distribution of results for the laboratory tests most commonly used in diagnosis and the study of host immune response to *L. chagasi* (serology, parasite identification in tissues and leishmanin skin test). We examined these parameters in a population of stray dogs collected from the streets of Jequié (a visceral leishmaniasis-endemic area in the state of Bahia, Brazil) [18]. As shown in **Figure 1**, more than half (51%) of the animals were positive for only one of these tests. Among the animals with more than one positive test, most had both positive serology and positive spleen culture. Although it is a non-specific symptom, as most of the clinical signs of canine visceral leishmaniasis are, emaciation is one of the most frequent clinical signs of visceral leishmaniasis in dogs [19, 20]. Therefore, we compared the distribution of emaciation in this group of animals according to the positivity of the different tests. As can be seen in the **Figure 2**, the proportion of animals with emaciation was greater in animals with positive spleen culture and with positive serology against *Leishmania* than in animals with negative results for these tests [18]. Conversely, the frequency of emaciation was lower in animals with a positive reaction to the leishmanin skin test (LST) than in animals with negative results. These data confirmed observations by other authors that positive spleen parasitism and positive serology are indicative of susceptibility to visceral leishmaniasis, and that positive LST used in association with other parameters is indicative of protection against the disease [21-23]. Using these parameters, we distributed the animals into four working groups according to their potential susceptibility to visceral leishmaniasis and compared the frequency of spleen histological changes in these groups. Because parasite identification in tissues is the most reliable sign of active infection, we used this parameter together with the LST in the definition of these groups, as follows: (a) Potentially resistant to visceral leishmaniasis: animals with a positive LST and spleen culture negative for *Leishmania*; (b) Potentially susceptible to visceral leishmaniasis: animals with a negative LST and spleen culture positive for *Leishmania*; (c) Infected with undefined susceptibility status, animals with both a positive LST and spleen culture positive for *Leishmania*; and (d) Non-infected: animals with negative spleen cultures, a negative LST, and without anti-*Leishmania* antibody activity in the serum as measured by ELISA [24]. The animals in the potentially susceptible group were more frequently emaciated (60%) than the animals in the other groups (27%, χ^2-test, P = 0·02). Only this group of animals had anti-*Leishmania* antibody activity in the serum significantly higher than that observed in the control group [24]. The animals in this potentially susceptible group also had a greater

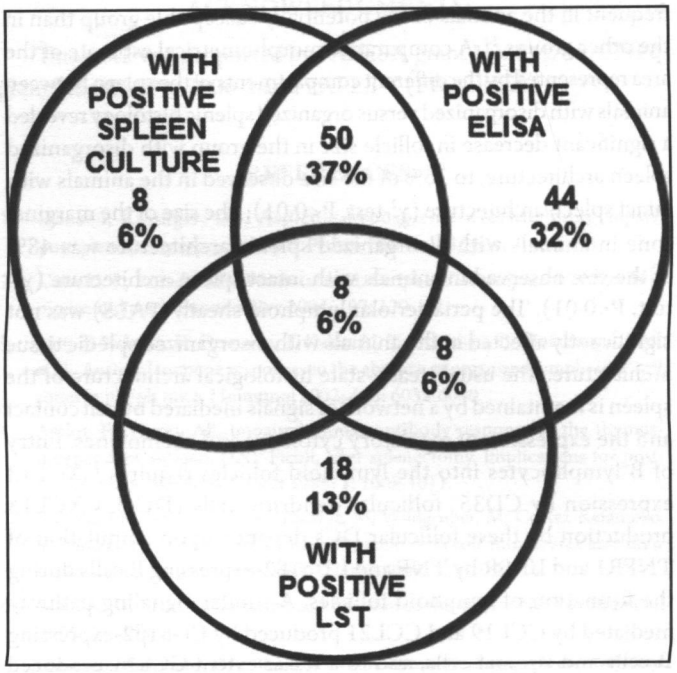

FIGURE 1 - Distribution of positive results on commonly used visceral leishmaniasis tests in a population of stray dogs from an endemic area of the disease (LST = leishmanin skin test).

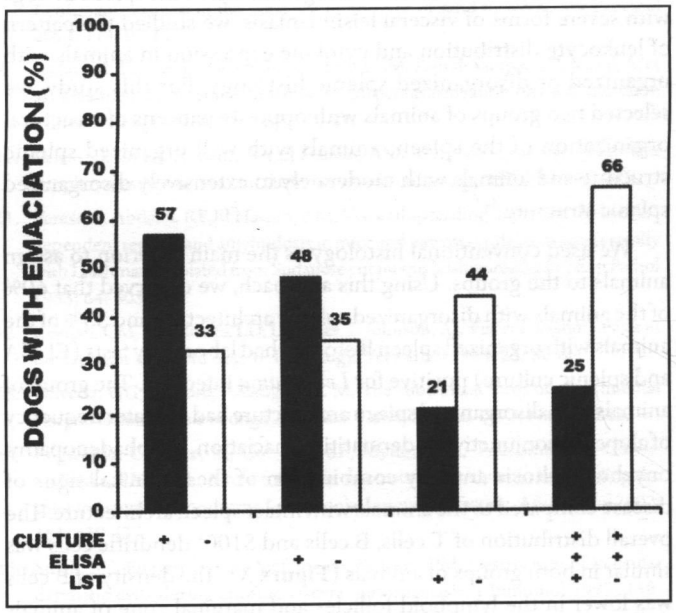

FIGURE 2 - Association between emaciation and positive results on tests commonly used to detect infection and susceptibility to visceral leishmaniasis in dogs (LST = leishmanin skin test).

frequency of perisplenitis (72%, χ^2-test, P < 0.01), granuloma (28, χ^2-test, P = 0.01), and amastigotes in the spleen (40%, χ^2-test, P<0.01) than the other groups. Furthermore, a variety of changes in the white pulp of the spleen, ranging from hyperplasia to atrophy, were observed in association with disorganization of the splenic histological architecture. Hyperplasia was more frequent in the animals of the potentially resistant group. Atrophy of the lymphoid follicles (20/25, χ^2-test, P<0.01) and of the marginal zone (15/25, χ^2-test, P<0.01) associated with architectural disorganization of the spleen histology (14/25, χ^2-test, P<0·01), which included loss of definition of the compartments of the white pulp and partial replacement of the usual leukocytes present in the white and red pulp of the spleen by plasmacytoid and plasma cells, was more

frequent in the animals of the potentially susceptible group than in the other groups [24]. A comparative morphometrical estimate of the area represented by the different compartments of the spleen between animals with disorganized versus organized splenic histology revealed a significant decrease in follicle size in the group with disorganized spleen architecture, to 36% of the size observed in the animals with intact spleen architecture (χ^2-test, P<0·01); the size of the marginal zone in animals with disorganized splenic architecture was 48% of the size observed in animals with intact spleen architecture (χ^2-test, P<0·01). The periarteriolar lymphoid sheath (PALS) was not significantly affected in the animals with disorganized splenic tissue architecture. The usual steady-state histological architecture of the spleen is maintained by a network of signals mediated by cell contact and the expression of regulatory cytokines and chemokines. Entry of B lymphocytes into the lymphoid follicles requires CXCL13 expression by $CD35^+$ follicular dendritic cells (DCs). CXCL13 production by these follicular DCs depends upon stimulation of TNFR1 and LT-βR by TNF and LT-α1β2-expressing B cells during the formation of lymphoid follicles. A similar signaling pathway mediated by CCL19 and CCL21 produced by LT-α1β2-expressing B cells and stromal cells, and to a lesser extent CCL21 produced by DCs, is involved the recruitment of T lymphocyte to the T cell areas of the spleen (reviewed by [6]). To see which signaling pathways were involved in the structural disorganization of the spleen in dogs with severe forms of visceral leishmaniasis, we studied the pattern of leukocyte distribution and cytokine expression in animals with organized or disorganized splenic histology. For this study, we selected two groups of animals with opposite patterns of structural organization of the spleen: animals with well-organized splenic structure and animals with moderately to extensively disorganized splenic structure [24].

We used conventional histology as the main criterion to assign animals to the groups. Using this approach, we observed that 60% of the animals with disorganized splenic architecture and 33% of the animals with organized spleen histology had laboratory tests (ELISA and splenic culture) positive for *Leishmania* infection. The group of animals with disorganized spleen architecture had a greater frequency of alopecia, conjunctivitis, dermatitis, emaciation, lymphadenopathy, onychogryphosis and any combination of these clinical signs of disease compared to the animals with intact spleen architecture. The overall distribution of T cells, B cells and $S100^+$ dendritic cells was similar in both groups of animals (**Figure 3**). The density of B cells was lower in the lymphoid follicles and marginal zone of animals with disorganized splenic architecture (13931 ± 7790 cells/mm^2 and 11876 ± 2145 cells/mm^2, respectively) than in the animals with intact splenic architecture (37651 ± 8694 cells/mm^2 and 16552±3583 cells/mm^2, Mann-Whitney, P < 0.001 and P < 0.05, respectively). The density of DCs was greater in the lymphoid follicles of animals with disorganized splenic architecture (1994 ± 533 cells/mm^2) than in the animals with intact splenic architecture (1364 ± 533 cells/mm^2, Mann-Whitney, P < 0.04). The estimated total number of cells was lower for T lymphocytes (360 ± 295 cells), B lymphocytes (618 ± 519 cells) and $S100^+$ DCs (97 ± 56 cells) in the follicles and for B lymphocytes in the marginal zone (1774 ± 1013 cells) in animals with disorganized spleen architecture, compared to animals with undisturbed spleen histology (773 ± 469 cells; 5083 ± 2356 cells; 183 ± 91 cells; 3687 ± 1188 cells, Mann-Whitney, P < 0.05; P < 0.01; P < 0.05; P < 0.01, respectively). Among the tested cytokines, only LTα (1.4 ± 1.3) and CXCL13 (0.5 ± 0.3) showed lower expression

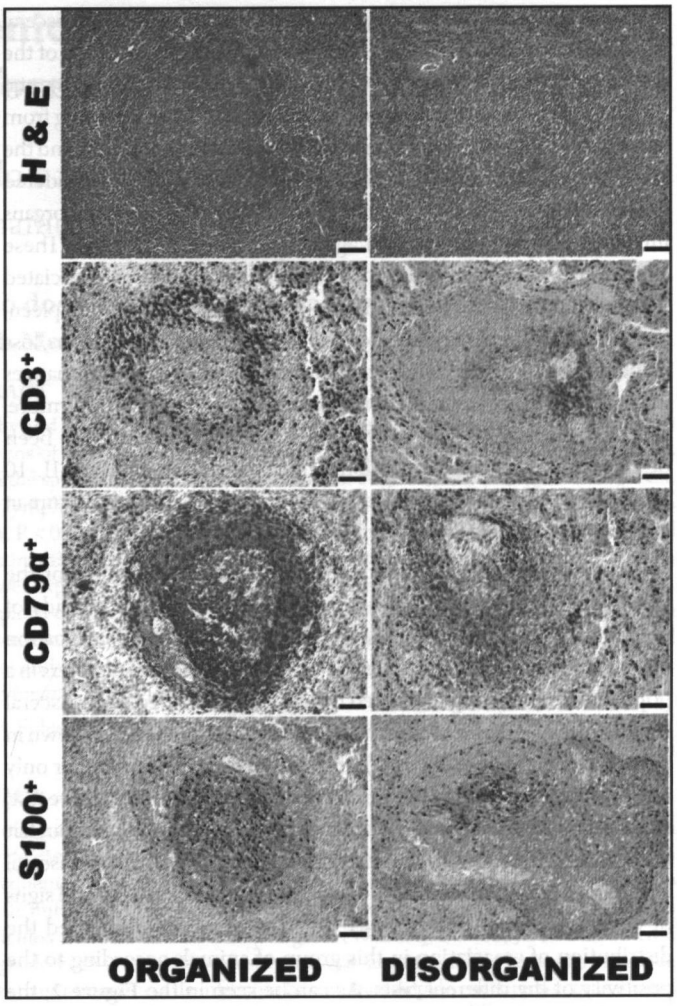

FIGURE 3 - Distribution of leukocyte populations in the spleen of dogs with organized and disorganized histological architecture.

(relative to constitutive 18S ribosomal RNA expression) in the spleens of animals with disorganized splenic architecture compared to animals with normal splenic architecture (2.7 ± 2.2 and 2.4 ± 2.4, P < 0.05 and P < 0.01, respectively). The expression of other tested cytokines such as TNF, IL-10, IFNγ, TGFβ, CCL21 and CCL19 did not differ between the groups.

Taken together, the data that we present here support the following conclusions: (1) dogs with markers of susceptibility to LV have architectural disorganization of the splenic tissue; (2) other, possibly infectious conditions, besides visceral leishmaniasis also induce architectural disorganization of the splenic tissue in dogs from an endemic area of visceral leishmaniasis; (3) the architectural disorganization of the splenic tissue is uneven among the different compartments of the spleen, affecting mainly the marginal zone and lymphoid follicles; (4) there is a redistribution of leukocyte populations associated with architectural disorganization of the splenic tissue; and (5) the architectural disorganization of the splenic tissue in dogs is associated with aberrant expression of cytokines involved in the structuring of the follicles and marginal zone of the spleen, such as LTα and CXCL13. As we reviewed above, in an experimental model of murine visceral leishmaniasis, infection with *Leishmania* by itself could result in disorganization of spleen histological architecture. In stray dogs from an endemic area of visceral leishmaniasis, other infections can further contribute to structural changes in the spleen.

We are now interested in the potential consequences of such disorganization of spleen microenvironments in the course of visceral leishmaniasis. It has been shown that human beings with visceral leishmaniasis have a greater frequency of bacterial infections of the skin, respiratory tract, oral cavity and middle ear compared to the general population [25-27]. As we reviewed above, the spleen plays an important role in the defense against systemic viral and bacterial infection. Much of the role played by the spleen in the defense against infections is dependent upon cell interactions that occur in the microenvironments of the marginal zone and the lymphoid follicles. Our study and research by other groups show that these microenvironments are disrupted in dogs with severe forms of visceral leishmaniasis. In order to investigate the potential association between changes in splenic architecture and the course of visceral leishmaniasis, we initiated a study of the frequency of co-infections in dogs with natural infection with *L. chagasi*, and the association of these infections with the presence of structural disorganization of the spleen. Preliminary data from this study show that bacterial infection is frequent in the population of stray dogs in an endemic area of visceral leishmaniasis. *Streptococcus* and *Staphylococcus* are the main microorganisms infecting the skin and eyes of these animals (**Table 1**). There is a greater frequency of bacterial skin infections in animals with spleen cultures positive for *Leishmania* (9/19, 47%) than in animals with negative cultures (7/34, 21%) (**Table. 2**). It is interesting to note that, although eye infection also tends to be more frequent in animals with *Leishmania*-positive spleen cultures than in animals with negative cultures, the frequency (26%) of blood infection with different bacteria (**Table 1**) was similarly high in both animal groups (**Table 2**). The analysis of these observations on bacterial infection together with the data on clinical signs of disease and the study of spleen histology in these animals are now under way in our laboratory.

TABLE 1 - Leishmania and bacterial infections in different tissue of dogs from an endemic area of visceral leishmaniasis.

Microorganism	Spleen	Eyes	Skin	Blood
Leishmania	20/61	ND	ND	ND
Staphylococcus aureus		4/68	3/68	3/61
Streptococcus b-hemolytic		12/68	3/68	0/61
Staphylococcus + Streptococcus		1/68	1/68	0/61
Proteus mirabilis		1/68	1/68	1/61
Pseudomonas aeruginosa		1/68	0/68	0/61
Actinobacter spp		0/68	1/68	1/61
Enterobacter spp		1/68	0/68	0/61
Klebsiella pneumoniae		1/68	0/68	0/61
Salmonela spp		0/68	0/68	2/61
Anaerobium		0/68	0/68	3/61
Yeast		ND	ND	0/61
Undefined		0/68	0/68	6/61

TABLE 2 - Frequency of bacterial infection in dogs with spleen culture positive for *Leishmania*.

	Spleen culture for leishmania isolation	
Tissue	Negative (%)	Positive (%)
Eyes	20/34 (59)	15/19 (79)
Skin	7/34 (21)	9/19 (47)*
Blood	9/35 (26)	5/19 (26)

*χ^2-test, P = 0.04

ACKNOWLEDGMENTS:

This work was supported by FAPESB, grant No. 434/05, CNPq grant No. 301882/2006-1 and PAPES V (FIOCRUZ).

REFERENCES:

1. Hansen, K,Singer, DB, Asplenic-hyposplenic overwhelming sepsis: postsplenectomy sepsis revisited. Pediatr Dev Pathol 2001; 4: 105-121.
2. Carsetti, R, Rosado, MM,Wardmann, H, Peripheral development of B cells in mouse and man. Immunol Rev 2004; 197: 179-191.
3. Junt, T, Nakano, H, Dumrese, T, Kakiuchi, T, Odermatt, B, Zinkernagel, RM, et al., Antiviral immune responses in the absence of organized lymphoid T cell zones in plt/plt mice. J Immunol 2002; 168: 6032-6040.
4. Amlot, PL,Hayes, AE, Impaired human antibody response to the thymus-independent antigen, DNP-Ficoll, after splenectomy. Implications for post-splenectomy infections. Lancet 1985; 1: 1008-1011.
5. Swirski, FK, Nahrendorf, M, Etzrodt, M, Wildgruber, M, Cortez-Retamozo, V, Panizzi, P, et al., Identification of splenic reservoir monocytes and their deployment to inflammatory sites. Science 2009; 325: 612-616.
6. Mebius, RE,Kraal, G, Structure and function of the spleen. Nat Rev Immunol 2005; 5: 606-616.
7. Carneiro, JJ,Donald, DE, Blood reservoir function of dog spleen, liver, and intestine. Am J Physiol 1977; 232: H67-72.
8. Paranhos-Silva, M, Oliveira, GG, Reis, EA, de Menezes, RM, Fernandes, O, Sherlock, I, et al., A follow-up of Beagle dogs intradermally infected with Leishmania chagasi in the presence or absence of sand fly saliva. Vet Parasitol 2003; 114: 97-111.
9. Badaro, R, Falcoff, E, Badaro, FS, Carvalho, EM, Pedral-Sampaio, D, Barral, A, et al., Treatment of visceral leishmaniasis with pentavalent antimony and interferon gamma. N Engl J Med 1990; 322: 16-21.
10. Veress, B, Omer, A, Satir, AA,El Hassan, AM, Morphology of the spleen and lymph nodes in fatal visceral leishmaniasis. Immunology 1977; 33: 605-610.
11. Veress, B, Abdalla, RE,El Hassan, AM, Visceral spreading depletion of thymus-dependent regions and amyloidosis in mice and hamsters infected intradermally with Leishmania isolated from Sudanese cutaneous leishmaniasis. Br J Exp Pathol 1983; 64: 505-514.
12. Keenan, CM, Hendricks, LD, Lightner, L,Johnson, AJ, Visceral leishmaniasis in the German shepherd dog. II. Pathology. Vet Pathol 1984; 21: 80-86.
13. Oliveira, GG, Santoro, F,Sadigursky, M, The subclinical form of experimental visceral leishmaniasis in dogs. Mem Inst Oswaldo Cruz 1993; 88: 243-248.
14. Engwerda, CR, Ato, M, Cotterell, SE, Mynott, TL, Tschannerl, A, Gorak-Stolinska, PM, et al., A role for tumor necrosis factor-alpha in remodeling the splenic marginal zone during Leishmania donovani infection. Am J Pathol 2002; 161: 429-437.
15. Smelt, SC, Engwerda, CR, McCrossen, M,Kaye, PM, Destruction of follicular dendritic cells during chronic visceral leishmaniasis. J Immunol 1997; 158: 3813-3821.
16. Benedict, CA, De Trez, C, Schneider, K, Ha, S, Patterson, G,Ware, CF, Specific Remodeling of Splenic Architecture by Cytomegalovirus. PLoS Pathog 2006; 2: e16.
17. Ato, M, Stager, S, Engwerda, CR,Kaye, PM, Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. Nat Immunol 2002; 3: 1185-1191.
18. Dos-Santos, WL, Jesus, EE, Paranhos-Silva, M, Pereira, AM, Santos, JC, Baleeiro, CO, et al., Associations among immunological, parasitological and clinical parameters in canine visceral leishmaniasis: Emaciation, spleen parasitism, specific antibodies and leishmanin skin test reaction. Vet Immunol Immunopathol 2008; 123: 251-259.
19. Ciaramella, P, Oliva, G, Luna, RD, Gradoni, L, Ambrosio, R, Cortese, L, et al., A retrospective clinical study of canine leishmaniasis in 150 dogs naturally infected by Leishmania infantum. Vet Rec 1997; 141: 539-543.
20. Barrouin-Melo, SM, Larangeira, DF, de Andrade Filho, FA, Trigo, J, Juliao, FS, Franke, CR, et al., Can spleen aspirations be safely used for the parasitological diagnosis of canine visceral leishmaniosis? A study on assymptomatic and polysymptomatic animals. Vet J 2006; 171: 331-339.

21. Pinelli, E, Killick-Kendrick, R, Wagenaar, J, Bernadina, W, del Real, G,Ruitenberg, J, Cellular and humoral immune responses in dogs experimentally and naturally infected with Leishmania infantum. Infect Immun 1994; 62: 229-235.

22. da Silva, VO, Borja-Cabrera, GP, Correia Pontes, NN, de Souza, EP, Luz, KG, Palatnik, M, et al., A phase III trial of efficacy of the FML-vaccine against canine kala-azar in an endemic area of Brazil (Sao Goncalo do Amaranto, RN). Vaccine 2000; 19: 1082-1092.

23. Reis, AB, Teixeira-Carvalho, A, Vale, AM, Marques, MJ, Giunchetti, RC, Mayrink, W, et al., Isotype patterns of immunoglobulins: hallmarks for clinical status and tissue parasite density in Brazilian dogs naturally infected by Leishmania (Leishmania) chagasi. Vet Immunol Immunopathol 2006; 112: 102-116.

24. Santana, CC, Vassallo, J, de Freitas, LA, Oliveira, GG, Pontes-de-Carvalho, LC,dos-Santos, WL, Inflammation and structural changes of splenic lymphoid tissue in visceral leishmaniasis: a study on naturally infected dogs. Parasite Immunol 2008; 30: 515-524.

25. Andrade, TM, Carvalho, EM,Rocha, H, Bacterial infections in patients with visceral leishmaniasis. J Infect Dis 1990; 162: 1354-1359.

26. Guerreiro, J, Ribeiro, S, Carvalho, EM, Badaro, R,Rocha, H, [Bacterial infection in patients with visceral leishmaniasis]. Mem Inst Oswaldo Cruz 1985; 80: 447-452.

27. Kadivar, MR, Kajbaf, TZ, Karimi, A,Alborzi, A, Childhood visceral leishmaniasis complicated by bacterial infections. East Mediterr Health J 2000; 6: 879-883.