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Expression of Spleen Structural Components of FeLV-Positive and FeLV-Negative Cats (*Felis catus*)

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ABSTRACT

Considering the spleen great importance for the immune system and the role of this organ in response to Feline Leukemia Virus (FeLV) infection in response to Feline Leukemia Virus (FeLV) infection, the objective of this study was to evaluate spleen histomorphometric changes, as well as its weight and volume. Furthermore, lymphocytes were quantified and the VEGF system expression was analyzed, especially its VEGF-A protein, which is part of physiological angiogenesis in different body organs. Spleens of infected and non-infected cats of six months to three years old were collected and used for confection of histological slides, which passed through the coloration process and immunohistochemistry technique. We observed state of splenomegaly, however, there were no statistically significant histomorphometric changes. Presence of lymphocytes was significantly reduced in the diseased group when compared with the control. On the other hand, there was a statistically significant increase in the VEGF-A protein expression.

Key words: Feline leukemia virus, physiology, spleen tissue, VEGF system

INTRODUCTION

The most important spleen activities are the immuno and phagocytic functions. Among these, we may cite blood filtration and storage, destruction of old erythrocytes, formation of blood cells along fetal life and organism immune defense via antibody formation and proliferation of T and B cells (Wilkins, 2002).

The organ is surrounded by a fibrous capsule of connective tissue that emits trabeculae, which are connective tissue projections towards the parenchyma (Mebius and Kraal, 2005). The splenic parenchyma is divided into three compartments (Benter *et al*., 2011). The red pulp consists of a three dimensional meshwork of splenic cords and venous sinuses. Apart from reticular cells, splenic cords have macrophages, monocytes, lymphocytes, plasmacytes, erythrocytes, platelets and granulocytes. The red pulp occupies most of the organ parenchyma where blood elements are concentrated, that is why it is considered a reservoir of erythrocytes, which are released in the splenic contraction (Cesta, 2006). The white pulp is organized as a

lymphoid coating with B and T cell compartments, surrounded by an arterial vascular branch and resembling the lymph node structure (Mebius and Kraal, 2005).

The so-called marginal zone represents the third compartment, which separates the red from the white pulp (Benter *et al*., 2011). The Feline Leukemia Virus (FeLV) is an important worldwide distributed retrovirus that affects, mainly, domestic cats. The infection, in general, occurs in the oropharynx, where the virus infects lymphocytes, thus reaching the bone marrow (Lutz *et al*., 2009). Infected cells carry the viral agent to other target tissues, such as thymus, spleen and lymph nodes (Levy, 2004). Clinical signs associated with FeLV infection may be classified as tumors, immunosuppression, hematologic disorders, immune-mediated diseases and other syndromes (Hartmann, 2011).

The objective of this study was to describe the changes promoted by FeLV infection, as well as functional and structural modifications that affected the spleen. Spleens of FeLV-positive (Fp) and FeLV-negative (Fn) animals were used to quantify densities of red pulp, white pulp, connective tissue, lymphocytes and VEGF-A.

MATERIALS AND METHODS

Nine domestic cats (*Felis catus domesticus*), that is, four males and five females, were evaluated. They were divided into two groups: the first, FeLV-negative (Fn), comprised four females and one male, totaling five animals; the second group, FeLV-positive (Fp), consisted of three males and one female, therefore, four. All of them were in the Veterinary Hospital belonging to University of Brasília. The study was approved by the Animal Use Ethics Committee of the University of Brasília (≠109508/2011).

The animals used on Fp group were standardized presenting common FeLV symptomatology, i.e., hyporexia and prostration. They also had severe anemia, with packed cell volume ranging from 6-9%. Animals were six months to three years old. All of them were euthanized at the owner's discretion because of bad prognosis and animal physical condition. Fn group was around six months to two years old and died because of other reasons, which did not derail their inclusion in the FeLV-negative group, such as surgical and postoperative complications. All animals were submitted to SNAP FeLV/FIV Combo Test (INDEXX Laboratories) to prove their FeLV positivity or negativity.

Spleen weight was estimated with the aim of a precision scale. Organ volumes, for each group, were established according to the volumetric determination model. For the microscopic analysis, the spleen was entirely segmented, random fragments were fixed in 10% formaldehyde aqueous solution, submitted to conventional histological technique and included in paraffin blocks for later tissue cleavage by hand microtome (Spencer-Lens Co.) into fragments of 4 μm width. Slides were then stained with hematoxylin-eosin for quantification of the area occupied by both white and red pulps. For quantification of the connective tissue, slides were stained with picrosirius red.

For quantification in the spleen structure, organ fragments from both groups were quantified via conventional immunohistochemistry, using CD20 monoclonal primary antibodies (M774, DAKO) and the universal secondary antibody (Streptavidin Biotin, LSAB+System-HRP, Dako, Glostrup, Denmark). Photomicrographs of five random fragment areas were obtained with the aim of an optical microscope BX51 Olympus® coupled to Prog Res® Capture Pro 2.5 software for image capture and analysis. The area occupied by white and red pulps and lymphocytes, was

quantified by a point system using STEPanaizer© software. For determination of the connective tissue proportion in the organ and VEGF-A protein arrangement, images were captured and analyzed by Image-Pro Plus 6.0® software.

Statistical analysis: Data was presented as means and standard deviations, submitted to Kolmogorov-Sminorv normality test and compared by Mann Whitney test, besides Pearson and Spearman correlation (GraphPad Prism 2.6 for Windows, GraphPad Software, San Diego, CA, USA), considering $p<0.05$ as statistically significant.

RESULTS

Weight and volume of spleens were, respectively, a mean of 9.44±1.96 g and 10±2.58 mL^3 for Fp and 17.83±1.66 g and 17.25±1.92 mL^3 for Fp. Volume and weight of Fn spleens were greater than Fn ones ($p = 0.041$ for weight and $p = 0.049$ for volume), what characterizes splenomegaly. The statistics analysis demonstrated that only the correlations between white pulp of both groups and between white pulp of Fn group and red pulp of Fp group were considerated significant. As shown on the Table 1, the r values found in the correlation tests didn't showed a pattern. However, weight, volume and connective tissue of Fn group revealed a strong positive relationship when compared to volume of Fp group. The other correlations were shown to be weak or moderated.

Presence of connective tissue (Fig. 1-3) comprised a mean of 13.28±1.69% for Fn and 12.11±2.51% for Fp (Fig. 1a and 2c-d). As there was no variation of connective tissue amounts between groups, the results suggest that the disease itself did not cause any organ remodeling.

The white pulp (Fig. 2) in Fn spleens was, on average, 6.02±2.02%; for Fp animals, it was 8.38±2.92% (Fig. 1b). For the evaluated cats, the quantitative analysis showed that FeLV infection was not able to promote an immune response that generated changes in the white pulp density.

Quantification of the red pulp presented a mean of 73.08±3.13% for Fn and 73.85±4.53% for Fp, regardless the intense anemia due to FeLV infection (Fig. 1c). Such condition did not change the red pulp normal structural conformation (Fig. 2) or connective tissue density of cat spleens, with no differences between groups.

There was statistical difference ($p = 0.0317$) between groups for lymphocyte (Fig. 3) quantification, with means of 13.14±1.35% for Fn and 19.14±3.85% for Fp (Fig. 1d).

There was also variation in the VEGF-A system (Fig. 3b), with statistical difference ($p = 0.0018$) between groups. The mean density, observed via immunohistochemistry, was 1.19±0.60% for Fn and 4.05±1.56% for Fp (Fig. 1e).

Table 1: Pearson and Spearman correlation coefficient between the structures of FeLV-negative and FeLV-positive groups

	FeLV-positives				
FeLV-negatives	Weight	Volume	Connective tissue	White pulp	Red pulp
Weight	0.3162	0.9487	0.3714	-0.02857	0.1429
Volume	0.5000	0.8333	0.5296	-0.02942	0.2648
Connective tissue	0.3162	0.9487	0.1287*	-0.006053	0.1672*
White pulp	0.3162	0.1054	-0.1176	0.4447	-0.4566
Red pulp	0.5000	-0.5000	0.2202	-0.1820	-0.0744

*Pearson correlation

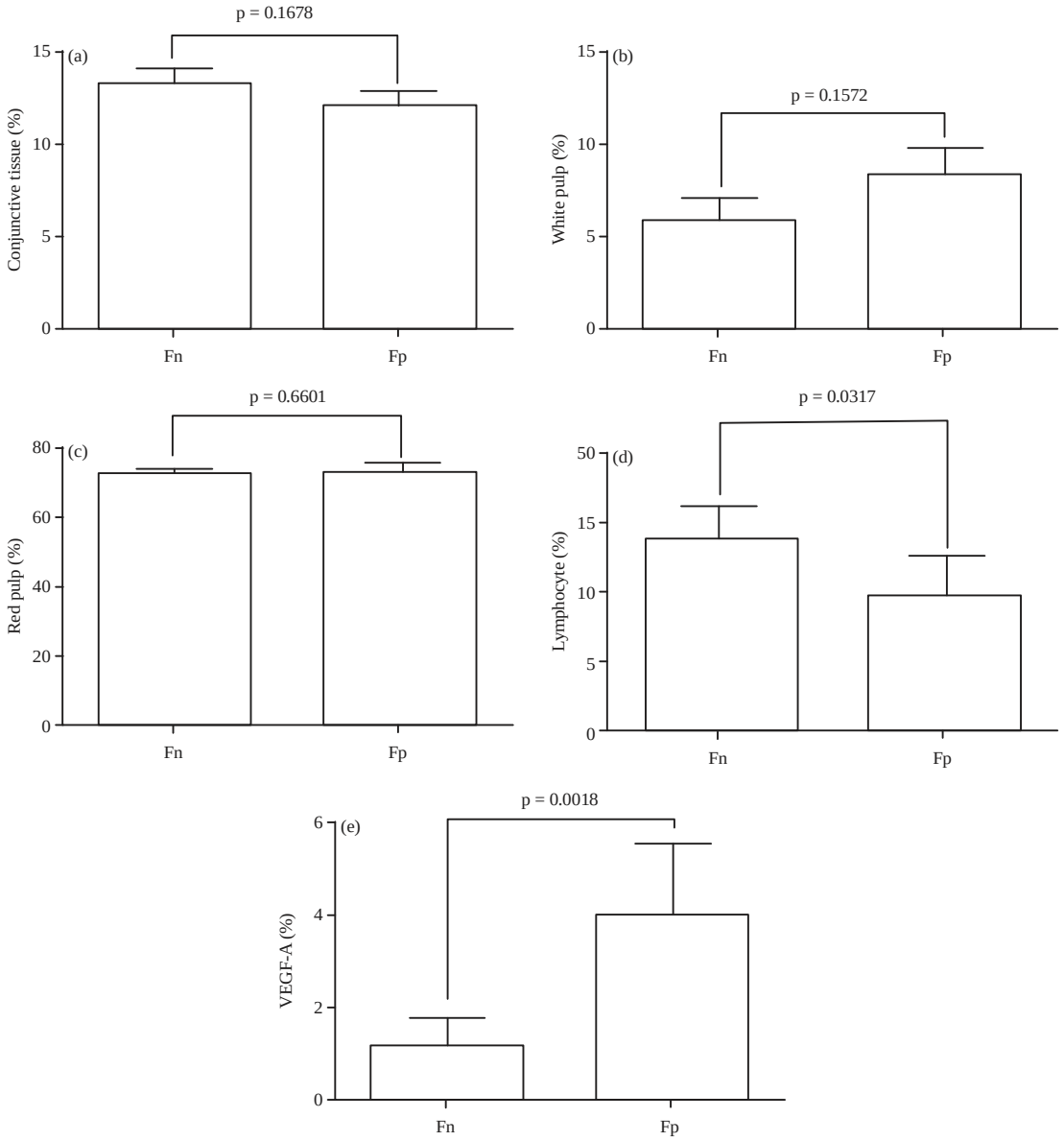

Fig. 1(a-e): Comparative graphs between control and diseased groups. Values represent Mean±SD The comparison between (d, e) Graphs (paired T and Kolmogorov-Smirnov tests) had $p<0.05$; $p>0.05$ was found for the comparison among (a, b) Graphs and (c) Paired T test

DISCUSSION

Considering the values of spleen volume and weight obtained for the evaluated groups (Fn and Fp), it was possible to understand that splenomegaly was characterized as the organ physiological response to the disease. Such response was expressed as an extra medullary hematopoiesis due to anemia presence (Hanson *et al*., 2001). Above all, it is possible to justify such change because splenomegaly may occur from the sixth week after inoculation with Feline Immunodeficiency Virus (FIV), thus reflecting the impairment of the monocyte-macrophage tissue (Zanutto *et al*., 2011).

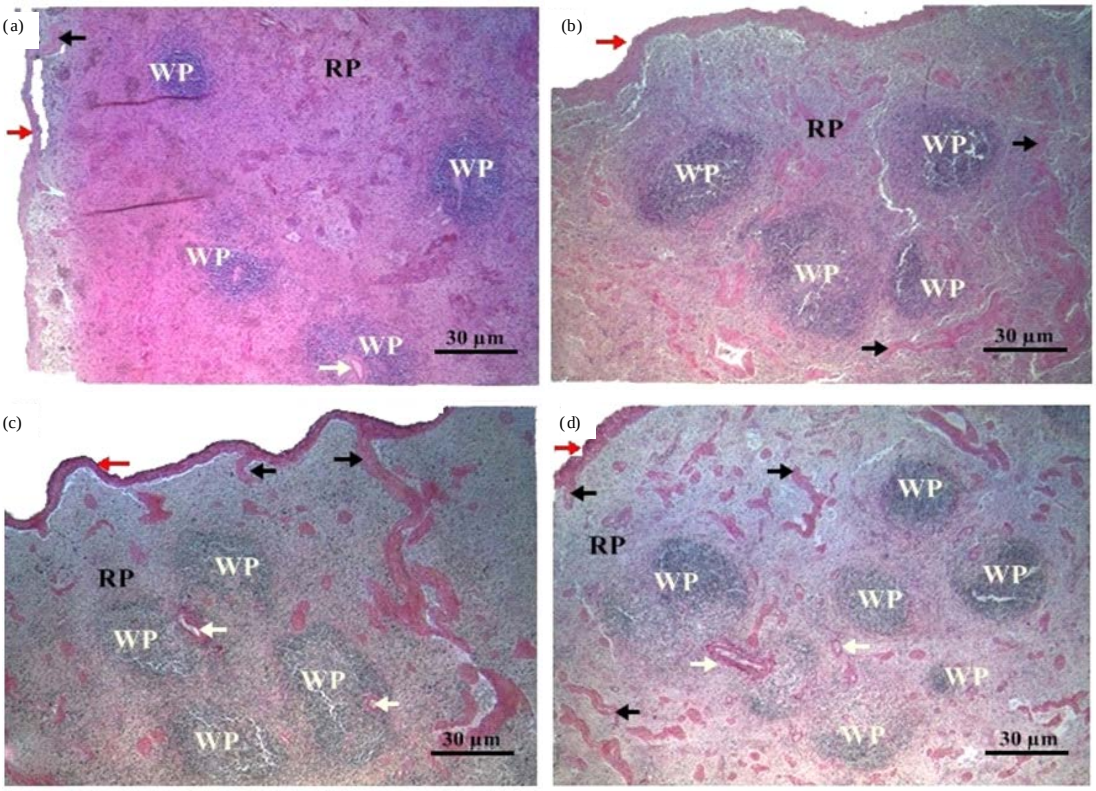

Fig. 2(a-d): (a, b) Photomicrographs of cat spleens stained with HE and (c, d) Picrosirius red, showing the RP: Red pulp, WP: White pulp, blood vessels (white arrow), connective tissue, composed of fibrous capsule (red arrow) and trabeculae (black arrow), (a, c) FeLV-negative and (b, d) FeLV-positive

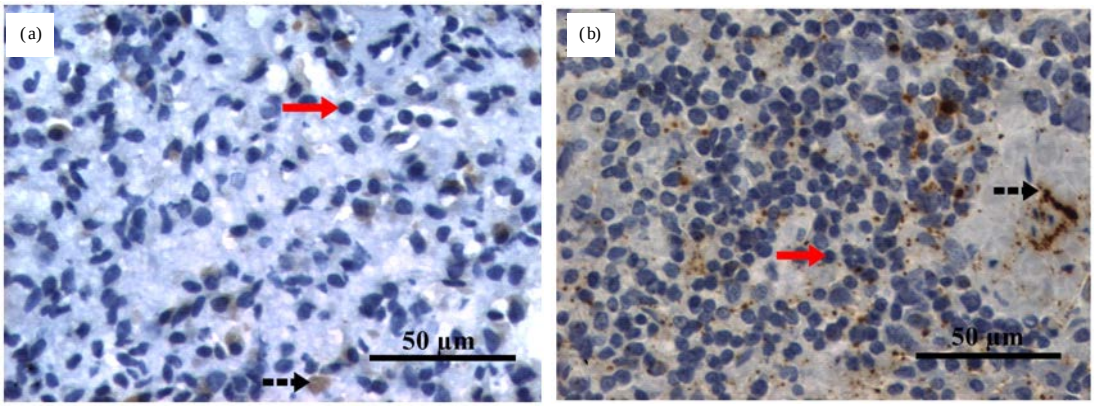

Fig. 3(a-b): Photomicrographs of the location of lymphocytes (red arrow) and VEGF-A protein (segmented arrow) via immunohistochemistry, (a) FeLV-negative and (b) FeLV-positive

Regarding the red pulp quantification, its predominance was found in rats and guinea pigs, occupying 65 and 54%, respectively (Furrianca *et al*., 2008). Although, hematopoietic stem cells are located in the murine spleen in contact with sinusoidal vessels in the red pulp (Kiel *et al*., 2005),

it was not known how these niches could contribute to either the hematopoiesis or nature of cell types involved in the response to the infection (O'Neill, 2012). Given the findings and coinciding with the present literature, it was possible to deduce that the red pulp is not a good measure of structural spleen changes.

Corroborating with our results, Furrianca *et al*. (2008) observed that the white pulp occupies a smaller area in spleens of rats (26%) and guinea pigs (22%); however, such percentage was higher than that found in this study. The acute immune response to antigens may generate an increased cellularity in the areas occupied by B cells in the white pulp (Elmore, 2006). Differently, periarteriolar lymphoid sheaths may have the cellularity either raised by lymphomas and leukemias, or reduced by exposition to radiation, virus and drugs (Elmore, 2006).

In this study, there was no distinction among different lymphocyte types. However, it has been observed a decrease of lymphocytes in Fp group, mainly when there is clinical symptomatology (Mansilla, 2007), what corresponds to the lymphocyte decrease found in spleens of the infected cats. It was possible to understand that the hyperplasia was not associated with the lymphocyte proliferation, whereas there was a relative reduction in the amount of these.

As it is known, tumor cells, such as the leukemia, release of Vascular Endothelial Growth Factor (VEGF) that promotes endothelial proliferation and increased vascularization in the bone marrow (Dias *et al*., 2002). The latter is a lymphoid organ like the spleen and may, perhaps, explain the VEGF-A protein increment in spleens of FeLV-infected cats.

Due to the VEGF-A increase and knowing that this protein is an important factor in angiogenesis during the pathogenic processes, being present in splenomegaly resultant of myeloid leukemia in humans, is understood that the increased weight and volume of the Fp group organs occurred due the possible neovascularization in response to the aforementioned extra medullary hematopoiesis (Liu *et al*., 2005). Medical parameters suggest the appearance of neovascularization supporting a possible hipertrophy characterized by the connective tissue proliferation.

Even though the FeLV infection causes significant systemic alterations in the spleen, as immunosuppression and anemia, this changes weren't capable to cause sufficient responses to modify the splenic architecture in the animals under study. Extra medullary erythropoiesis and decreased cellularity of white pulp could have been the spleen response against the condition and the consequent immunosuppression; however, this response wasn't capable to promote observable structural changes by histomorphometric analysis. The splenomegaly found in the Fp group could be a reflex against the pathologic conditions induced by FeLV infection.

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