Is the vascular growth curve of thymus of dogs (Canis familiaris) time-dependent?

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ABSTRACT

The characterization of formation, maintenance and involution of organs is crucial for understanding how the immune system develops, what mechanisms are ready to protect the newborn in the first microbial invasion and what can be done to enhance neonatal resistance to pathogens. Thymus is the modulator of the activity of other lymphoid organs, and knowledge of the mechanisms that trigger its development, maturation and involution, is essential for understanding the activity of the whole lymphoid system. It was used 36 thymses of domestic dogs (Canis familiaris) all mixed breed, aged between 30 days of development and 12 months of age, when it was evidenced, after this age, the involution of the organ. We measured the length, thickness and width of the organ using digital calipers, and the organ volume was estimated using the Cavalieri method. The fragments were subjected to conventional histological technique and cut to 4mm thickness with manual microtome for the preparation of slides. The quantitative analysis of the vessels was performed using physical disector stereological method and mechanism of expression of the VEGF system was the immunohistochemistry. The length, width, thickness and volume showed a gradual increase according to advancing age and development of fetuses and pups, coinciding with the development of animals and of the organ until six months of age, when it was evidenced, after this age, the involution of the organ. Stereological analysis of the thymus revealed, by means of densities of length, number and area of vessels, that the vasculature of this organ follows the same temporal behavior of structural development and involution. The immunohistochemistry indicated, for all groups analyzed, the expression of VEGF-A protein and Flt-1 and KDR receptors. In the stage of thymus involution, VEGF-A was expressed in a smaller amount and was best visualized in cell clusters formed by epithelial cells. Regarding the expression of Flt-1 and KDR, it was observed a behavior similar to that which occurred in the expression of VEGF-A in the stages of development and involution of the organ. The study of the thymus vasculature through stereological analysis and by examining the expression of the VEGF system contributed to better characterize the development of this organ in dogs with different ages, thereby confirming that the vascularization of thymus follows the time-dependent growth curve of the organ.

INTRODUCTION

The characterization of formation, maintenance and involution of organs is crucial for understanding how the immune system develops, what mechanisms are ready to protect the newborn in the first microbial invasion and what can be done to enhance neonatal resistance to pathogens. Thymus is the modulator of the activity of other lymphoid organs, and knowledge of the mechanisms that trigger its development, maturation and involution, is essential for understanding the activity of the whole lymphoid system (SINKORA et al., 2002). Angiogenic factors such as vascular endothelial growth factor (VEGF) is essential for the formation of the vascular bed tissue and for modulating functions not directly related to vascularization, such as cell migration and proliferation, hormone production and immunomodulation, which makes the knowledge of their expression in the thymus a key step towards the understanding of cell-cell interactions in the organ (KISELEVA et al., 2005).

Vascular endothelial growth factor (VEGF) is a mitogen specific for endothelial cells, responsible for the regulation of vascular development under normal and pathological conditions (DVORAK et al., 1991). The endothelial growth factor - A (VEGF-A) was the first and best described member of the VEGF family. Two VEGF-A receptors belonging to the family of tyrosine kinase receptors were identified: vascular endothelial growth factor receptor 1, also called fms-like tyrosine kinase receptor 1 (Flt-1) and vascular endothelial growth

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factor receptor 2 (VEGFR-2) or also called kinase insert domain receptor (KDR) (DE VRIES et al., 1992).

Knowledge of vascular behavior, of its structure, of the interference occurring in angiogenesis, in the development and involution of the thymus, as well as reflections such events determine on immunolabeling of animals may clarify several particularities not yet fully clarified. Among these autoimmune diseases, thymic tumors, persistent of the thymus and other immune-related impairments, which in short have great relevance for the understanding of physiology and pathophysiology of the thymus. Given these aspects, it is justifiable to study the relationship of the vascular behavior of the thymus during its development and involution.

The goal of this study was to determine the vascular behavior of the thymus during development and involution, through length density, area density, and total number of vessels, and to characterize the temporal protein expression of the VEGF system in the thymus of dogs of different age groups (fetuses with 30, 40, 50 and 60 days, puppies at 6 months and adults at 1 year).

MATERIAL AND METHODS

Thymuses were collected from 36 domestic dogs (Canis familiaris) at the Veterinary Hospital of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo (HOVET-FMVZ-USP), Center for Zoonosis Control - CCZ-SP and at neutering campaigns conducted in the city of São Paulo. All mixed breed, aged 30 days of development and 12 months of age, of both sexes in equal number. Fetuses with 30, 40, 50 and 60 days of development, puppies with 6 months and adults with 12 months of age were separated into six age groups. The choice of the age of the animals advocated covering initial, middle and final thirds of fetal development, as well as the start of puberty and adulthood. The study was approved by the Ethics Committee on Animal Use of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo, Protocol # 995/2006.

An incision in the costovertebral joints exposed chest cavity in order to locate the main branches of the left internal thoracic artery, which was isolated and cannulated. Lavage and fixation of the vascular system of the thymus was performed by perfusion with a solution containing phosphate buffered saline (PBS) and 2% heparin followed by modified Karnovsky's fixative solution.

Macroscopic analysis:

Measurements of length, thickness and width of the organ were taken using a digital caliper (Starrett®). The volume of the organ or reference volume (V_{ref}) was estimated using the Cavalieri method. The results of macroscopic analysis are shown in Figure 1.

Processing of samples:

The thymus was randomly fragmented and processed according to the modified orientator method, obtaining random and uniformly isotropic sections which were histologically processed for histochemistry or immunohistochemistry.

Fragments were fixed in formaldehyde aqueous solution (10%) for 24 hours, dehydrated in increasing concentrations of ethanol, cleared in xylol and impregnated and embedded. Later, they were cut to 4mm thickness with use of manual microtome (Leica RM 2125RT) for the preparation of the slides.

Stereological analysis:

Quantitative analysis of the vessels was performed on slides stained with hematoxylin & eosin, through the physical disector stereological method, related to the ConnEuler principle, employing the program STEPanizer (http://stepanizer.com/) to determine the densities of number, length and area from a test system. Stereological analysis results are shown in Figure 2 and Table 1.

Immunohistochemistry:

For the immunohistochemistry, slides were deparaffinized in xylene, rehydrated in decreasing ethanol solutions and rinsed in distilled water. For antigen recovery was used citrate buffer solution (pH = 6) in a water bath at 96°C. After washing in phosphate buffer solution (PBS), endogenous peroxidase was blocked with 4% volume 30 hydrogen peroxide solution, after a new wash in phosphate buffer solution (PBS), nonspecific cross-reactions the blocked using Protein Block (Dako, Glostrup, Denmark) in a moist chamber at room temperature, followed by further washing in PBS and incubation with primary antibody for 20 hours in a moist chamber at 4°C. The primary antibodies used for the VEGF system were VEGF-A (Polyclonal Rabbit, VEGF A20: sc-152, Santa Cruz Biotechnology Inc®), VEGF-C (Polyclonal Rabbit, Zymed Laboratories Inc., Santa Cruz, CA, USA), Flt-1 (Polyclonal Rabbit, Flt-1, C17: sc-316, Santa Cruz Biotechnology Inc®), KDR (Polyclonal Rabbit, Flk-1, C20: sc-315, Santa Cruz Biotechnology Inc®) and Flt 4 (Polyclonal Rabbit, Flt-4, C20: sc-321, Santa Cruz Biotechnology Inc®). After this period, we proceeded incubation of the universal secondary antibody (Streptavidin-Biotin Kit, LSAB+System-HRP, Dako, Glostrup, Denmark) for 45 minutes in a moist chamber at
room temperature. Finally, the slides were revealed in DAB (DAB Kit - diaminobenzidine, DAKO) and counterstained with hematoxylin. Then we proceeded new dehydration, clearing in xylene and mounting with coverslips glued with synthetic resin dissolved in toluene (Permament). As negative controls we used sections incubated with PBS in place of primary antibody. Positive controls were made using placental tissue of cattle and dogs with proven labeling for the antibodies used. Finally, the thymic tissue sections were observed under a Nikon Eclipse (E-800) microscope equipped with a video camera (CoolSNAP-Pro of Color®) and images were captured using the software Image-Pro Plus 4.5 (Media Cybernetics Inc., USA) for observation of the expression of the VEGF system during development and involution of the thymus as well as the locations of such cells. The results of immunohistochemistry are shown in Figures 3, 4 and 5.

**Statistical analysis:**
Data are presented as mean and standard deviation. Data were subjected to one-way ANOVA followed by post hoc Holm Sidak test (GraphPad Prism 2.6 for Windows, GraphPad Software, San Diego, CA, USA). P ≤ 0.05 was considered as statistically significant.

**Results:**

**Macroscopic analysis:**
The length, width, thickness and volume showed a gradual increase with advancing age and development of fetuses and pups, which coincided with the development of animals and organ until six months of age, when we observed, after this age, involution of the thymus (Figure 1).

![Fig. 1](image_url)

**Fig. 1:** Graphs with mean values and standard deviation of length (A), width (B), thickness (C) and volume (D) of the thymus of dogs in different age groups.

**Structural changes:**
In the thymus of animals with 30 days, the tissue was not organized into cortical and medullary regions. We observed small lobular structures surrounded by a capsule of connective tissue forming cell clusters or cell islands, without emergence of septa towards the interior of parenchyma. These cell islands had different sizes, in the younger group were irregular and smaller with young cells, but with the development, islands became larger and rounded, with a more differentiated cell population. Large vessels were observed in the interstitial region, in the connective tissue involving cell clusters and absent inside the parenchyma. We observed small cells, with dense staining without differentiation, migrating from large vessels into the thymic parenchyma. These cell clusters were composed mainly of epithelial cells of types 1, 2 and 3, and fewer immature thymocytes. In some cellular clusters, largest and most developed, was observed early formation of the thymic corpuscles. On the periphery of the clusters was observed predominance of type 1 epithelial cells smaller and more dense than types 2 and 3.

After 40 days of development, it was observed the emergence of septa from the connective tissue capsule penetrating the thymic parenchyma near the corticomedullary junction, dividing the tissue into small lobules. Along with septa we observed vascular and neuronal tissue penetrating inside the parenchyma. Medullary and cortical regions became more defined. It was observed the presence of some cellular clusters, similar to those of group I, however, they began to organize into cortical and medullary regions. Although some lobules have the cortical and medullary division, the medullary region was still small, while the cortical region was larger, with a large population of thymocytes. Thymic corpuscles were more clearly defined, as well as epithelial cells. On the periphery of the lobules we observed a concentration of type 1 epithelial cells. Cortical and medullary regions showed a dense population of thymocytes and lymphocytes. We observed small, undifferentiated, dense cells, migrating from vessels into the thymic parenchyma. There were a large number of small blood vessels inside the parenchyma and in the lobules that had the division of cortical and medullary regions. These vessels were concentrated in the corticomedullary junction and in the lobules that lacked this division, the same were scattered throughout the parenchyma.

At 50 days, each thymic lobe was coated by a thin capsule of connective tissue. It was formed thickenings at the junction points of the capsule with the vessels nourishing the thymus. The connective tissue emitted projections from the capsule, from which it numerous septa emerged, partially dividing the lobe forming the lobular structure. In the points of emergence of septa, blood vessels predominated, which in turn branched
towards the interlobular septa and in the thymic parenchyma itself. It was observed the presence of perivascular subcapsular epithelium delimiting the entire surface and perivascular spaces of the thymus, with a flattened and elongated appearance, dividing the space inside the thymus and the vascularized parenchyma. The thymic tissue showed well-defined cortical and medullary regions, and a more heterogeneous cell population, with many mitotic divisions and maturation of thymic corpuscles. The vessels of the parenchyma showed large caliber. Undifferentiated cells were observed migrating from vessels of the connective tissue into the parenchyma.

At 60 days, the thymus was completely developed, with all the cell types and full maturation of lymphocytes. Vessels distributed in the parenchyma had large caliber. Undifferentiated cells were observed migrating from vessels of the connective tissue towards the interior of the parenchyma.

In animals at 6 months of age, these divisions between cortical and medullary regions were observed, but irregularly, moreover, it was evidenced an increase of connective tissue and presence of adipose tissue, characteristics of the early stage of involution. There was a reduction in cell population, including lymphocytes, and cells were more spaced with wide intercellular space. Type 1 cells at the periphery of the lobules were no longer evident. Vessels showed smaller caliber and were not much evident inside the parenchyma.

In animals with one year of age, the adipose tissue became more evident with small islands of remaining thymic tissue. These islands had all cell populations as in a young animal, but in smaller numbers. The thymic corpuscles were still evident.

**Stereological analysis:**

The stereological analysis of the thymus showed, through the densities of length, number and area of vessels, that the vasculature of this organ follows the same temporal behavior of structural development and involution (Figure 2).

![Graphs with mean values and standard deviation of length density (A), number density (B) and area density (C) of vessels of the thymus of dogs in different age groups.](image)

**Table 1:** P-values of ANOVA with post hoc Holm Sidak test to check for statistical differences (*p*<0.005) in length density (Lv), area density (Sv) and number density (Nv) of vessels in thymuses at different ages.

<table>
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<th>Parameters</th>
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<th>30 days</th>
<th>45 days</th>
<th>50 days</th>
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<td></td>
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**Immunohistochemistry:**

In all groups, we examined the expression of protein VEGF-A, and of Flt-1 and KDR receptors. The positive reaction for VEGF-A (Figure 3A-F) was observed in a network of epithelial cells found in the subcapsular region, in epithelial cells isolated in the cortical area, and in almost all epithelial cells of the medullary region. All thymic corpuscles showed positive staining. In the stage of thymus involution, VEGF-A was expressed in lower proportions and was best visualized in cell clusters formed by epithelial cells (Figure 3F).
Fig. 3: Photomicrograph of the VEGF-A protein localization by immunohistochemistry in thymus of dogs. A: fetus at 30 days of development. B: fetus at 40 days of development. C: fetus at 50 days of development. D: newborn at 60 days. E: animal with six months of age. F: animal with one year of age. Arrow: epithelial cell; Star: medulla; Asterisk: blood vessel.

Flt-1 (Figure 4) and KDR (Figure 5) were expressed as a network of epithelial cells in the subcapsular region, and in all epithelial cells of the medullary region. All thymic corpuscles showed staining. In the stages of evolution and involution of the organ we observed a similar behavior to that occurred in the expression of VEGF-A.
Fig. 4: Photomicrograph of the Flt-1 protein localization in thymus of dogs by immunohistochemistry. A: fetus at 30 days of development. B: fetus at 40 days of development. C: fetus at 50 days of development. D: newborn at 60 days. E: animal with six months of age. F: animal with one year of age. Star: medulla; Asterisk: blood vessel Arrow: epithelial cell; Circle: thymic corpuscle.
Fig. 5: Photomicrograph of the KDR protein localization by immunohistochemistry in thymus of dogs. A: fetus at 30 days of development. B: fetus at 40 days of development. C: fetus at 50 days of development. D: newborn at 60 days. E: animal with six months of age. F: animal with one year of age. Star: medulla; Arrow: epithelial cell; Circle: thymic corpuscle, Diamond: thymic formation; Asterisk: blood vessel.

Discussion:
Macroscopically, there were no significant differences in morphologic or topographic patterns established for the thymus of the species studied (SILVA et al., 2001; LIMA et al., 2007). Particularly, there was an increase of the thymus volume by the method of Cavaliere during development, and a decrease, during involution (Figure 1). The increased volume of thymus, especially in the period near the birth, suggests a certain relationship with hormonal changes, as well as its full development during this period (OLSEN et al., 1994), suggests the occurrence of changes that are probably not related to sex, since in this study there were no statistical differences in morphometric and stereological analyses regarding this variable.

Thymus is a physiologically hypoxic organ, thus thymocytes and epithelial cells have a certain adaptation to this condition, which is essential to physiological thymic processes such as selection, maturation and differentiation of thymocytes. According to Hale et al. (2002) genes commonly responsive to hypoxia, like genes COX-2, BNIP3 and HMOX-1, are not activated when thymus cells are subjected to low concentrations of oxygen, which occurs in any tissue or cell line exposed to these conditions. Also according to these authors, even
with a PO2 increase, cells from different anatomical regions of the thymus continue exhibiting a positive reaction to pimonidazole (a marker for hypoxic cells) even when located nearby blood vessels, indicating the activation of intrinsic mechanisms of maintaining hypoxic state. It was not possible to fully elucidate the mechanisms involved. However, in the species studied, the data pointed to a temporal regulation of the thymus vasculature, suggesting fluctuation in the blood supply to the organ. Combining this finding with the values found for the stereological variables, when not completely disappeared, thymic vessels decreased their caliber and number (Figure 2).

The vascular and morphological characteristics, and age-related involution are somewhat similar in all mammals, but these aspects may vary according to the species and breed (SILVA et al., 2001; SOLAROVIC et al., 2006; BARROSO et al., 2012). Moreover, thymic vasculature is considered as the third anatomical compartment of the organ (CUDDIHY et al., 2009), thus playing an additional role in the development and involution of the thymus, still responding by the classic function of carrying the precursors of lymphocytes, derived from the bone marrow into the organ.

Compared to the organ of adult dogs, the murine newborn thymus was dominated by the cortex, with little medullary area and in addition, showing a poorly defined corticomedullary junction and the transition to the adult pattern occurred rapidly, in the first week of postnatal life (CUDDIHY et al., 2009). In dogs, characteristics similar to the histological standard of adult thymus were already present before birth, suggesting that such an arrangement can infer species-specific differences in the processes of proliferation, maturation and differentiation of thymocytes, as well as in other events related to cell-cell interactions in the organ.

In the present study, descriptions of the localization of proteins of VEGF and Flt-1 and KDR receptors were based on their observation both in stromal cells and in hematopoietic cells, although double labeling techniques have not been conducted during processing samples, which would allow the accurate identification of cell types and subtypes. Leukocyte lineage cells were particularly difficult to identify. Nevertheless, this limitation did not prevent identification of thymocytes and epithelial cells, which allowed for inferences on protein expression of VEGF system in the thymus of dogs.

In general, VEGF-A, KDR and Flt-1 follow the vascular curve of thymus, with increased expression during involution of the organ, which can be related to a period of hypoxia. During involution there is a decrease in blood support to the organ, thus promoting a medium conducive to hypoxia by increasing the expression of VEGF-A and its receptors resulting in marked reduction of thymopoiesis and decreasing the dense capillary network of the thymus (FERRARA, 1999; CUDDIHY et al., 2009).

VEGF system protein expression was observed in thymic epithelial cells and thymic corpuscles in all stages. This reveals that the protein expression of VEGF-A of dog thymus resembled the findings of Cimpean et al. (2008) in humans, which corroborated the hypothesis of active participation of thymic corpuscles in thymopoiesis. These same authors observed immunostaining for VEGF-A and its receptors KDR and Flt-1 in thymic corpuscles in 100% of the samples of human thymuses with ages ranging from 1 month to 50 years of life, under normal and pathological physiological conditions. And stated that under normal conditions, after birth, VEGF expression decreases rapidly, and the immunostaining of thymic corpuscles for this growth factor and its receptors supports the hypothesis that thymic corpuscles are active structures in the process of maturation and selection of lymphocytes in the postnatal period.

In this context, the study on the thymus vasculature through stereological analysis and by examining the expression of the VEGF system contributed to better characterize the development of this organ in dogs with different ages, therefore confirming that the vascularization of thymus follows the time-dependent growth curve of the organ.

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