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PhD THESIS

RESEARCHES CONCERNING THE MORPHOLOGY OF THE NICTITATING GLAND IN DOGS

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ABSTRACT

The nictitating gland of the dog (superficial/accessory of the third eyelid) is a compound tubuloacinar gland that eliminates the seromucous secretion product by exocytose (merocrine process), contributing to the aqueous fraction of the tear film (90% of the lachrymal volume). The nictitating gland is part of the nictitating membrane structure and its secretion product reaches at the base of nictitating-bulbar conjunctival fornix through multiple excretory ducts.

The nictitating gland of the dog is the second important gland after the main lachrymal gland regarding the secretion of the tear film, respectively its aqueous fraction. The aqueous fraction of the tear film has a thickness of 7 μm in dogs (external lipid fraction – 0,1 μm and internal mucosal fraction – 2 μm), being secreted by the main lachrymal gland at a rate of 61%, nictitating gland 35% and accessory conjunctival glands of Krause and Wolfring 3%.

The middle aqueous layer of the tear film has an active role in the lubrication and protection of the ocular surface. The middle aqueous layer removes the foreign bodies mislead on the surface of the conjunctiva or cornea, and it contains antibacterial factors, including immunoglobulins, corneal nutrients and growth factors involved in regeneration of the damaged cornea.

The secretory failure of the nictitating gland, with different etiologies, causes lack of lubrication of the cornea with exposure to biotic or inert aggressive agents, impossibility to initiate the defense processes due to antibacterial and growth factors failure, resulting in the emergence of the keratoconjunctivitis *sicca*, which can lead to blindness.

The scientific references on the nictitating gland morphology in dog is limited to general anatomical, histological and histochemical descriptions, histopathological descriptions related to prolapse of the nictitating gland and keratoconjunctivitis *sicca* and various tumors.

According to bibliographical sources, histological and histochemical complex studies (periodic acid Schiff, Alcian blue pH 2,5 and pH 1, PAS-Alcian blue pH 2,5, orcein-Alcian blue pH 2,5) on the nictitating gland (including its secretion product) and on the nictitating membrane in dogs, insufficiently clarified in national and foreign scientific literature, on a large number of

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individuals, have not been taken so far. Studies regarding organogenesis, histochemistry and micromorphometry on the developing nictitating gland and membrane in dog fetuses, histological autofluorescence studies of the nictitating gland, normal cytological and ultrastructure studies of the nictitating gland, all of these were not conducted so far. Statistical and micromorphometrical studies of the histological structures of the nictitating gland and membrane in order to establish a causal or a contributory relationship between different dimensions of these structures and epidemiological aspects, clinical and histopathology of the morbid processes of the nictitating gland and membrane has not been achieved so far. All these unknown facts represent the aim of these thesis.

The first part entitled *"Literature review"*, consists of two chapters that summarize the main bibliographic database of scientific literature describing both the eye annexes organogenesis in mammals (chapter I) and conformation, structure, vascularization and inervation of the annexes organs of the eyeball in domestic mammals (chapter II). The second part „*Personal researches*" consists of five chapters (chapter III – VII). Chapter III presents the aims and objectives of the thesis, chapters IV, V and VI present and describe the results of the researches, including materials and methods, discussions, interpretations, analysis and partial conclusions that are to be drawn from researches. In chapter VII, the final conclusions are presented and synthesize the conducted researches. A number of 152 figures, 75 tables and 209 bibliographic references illustrate the thesis.

Chapter IV, named „*Organogenesis of the nictitating gland and membrane in dog*" refers to harvesting the nictitating membrane from both eyes of 39 dog fetuses, male and female, belonging to different breeds, from 6 pregnant bitches, following hysterectomy by request of owners, at 6 fetal ages: 38 days old (six fetuses), 42 days old (nine fetuses), 45 days old (five fetuses), 49 days old (six fetuses), 53 days old (eight fetuses) and 58 days old (five fetuses).

From the serial histological sections were obtained permanent histological preparations stained with hematoxylin-eosin (HE) and hematoxylin-eosin-methilene blue (HEMB) in order to study the morphological details, and for various histochemical aspects, the used stains were: periodic acid Schiff (PAS, with Schiff's reagent prepared after the *de Tomasi* formula) (for neutral glycoproteins), PAS-Alcian blue pH 2.5 (for neutral and acid sialylated glycoproteins), Alcian blue pH 2.5 (for acid sialylated glycoproteins), Alcian blue pH 1 (for acid sulphated glycoproteins) and orcein-Alcian blue pH 2.5 (for sulphated and sialylated glycoproteins).

The initiating of the nictitating gland organogenesis in the canine fetuses 38 days old is underlined by the proliferation of the bulbar conjunctival epithelium in the mesenchyme underlying under the shape of glandular epithelial cords from which acinar cell mass are detached. Subsequently, the glandular formations resulted by their detachment from the

epithelial invagination and cords or the primary ducts (patent), are initially directed towards the bulbar and basal cartilage of the nictitating membrane (in fetuses aged 42 days) and later on the palpebral side (in fetuses aged 45 days).

In fetuses of 45 days old, the nictitating membrane cartilage has all the morphological structures presented in the hyaline cartilage of adult animals.

Starting with fetuses aged 45 days, is beginning the secretion of the nictitating glandular structures (primordial nictitating gland), which contains neutral glycoproteins and from 49 days fetal age and acid sialylated glycoproteins in smaller amount.

The cartilage of the nictitating membrane, respectively the cartilaginous matrix material of the canine fetuses aged between 42 and 58 days, contains sulphated and sialylated glycoproteins and less neutral glycoproteins.

The palpebral glandular structures develop in length, from 122 μm in fetuses aged 42 days to 1121.6 μm in fetuses aged 58 days.

The bulbar glandular structures grow in length, from 933.68 μm in fetuses aged 42 days to 2540.1 μm in fetuses aged 58 days.

The basal glandular structures grow in length, from 532.2 μm in fetuses aged 42 days to 988.71 μm in fetuses aged 53 days and 939 μm in fetuses aged 58 days.

The cartilage of the nictitating membrane shows an increase in length with the age of fetuses, from 2424.9 μm in fetuses aged 42 days to 4586.8 μm in fetuses aged 58 days, and its thickness varies very little from one age to another within the limits of 93.21 to 120.2 μm.

Chapter V, entitled „*Histology, histochemistry, autofluorescence, cytology and ultrastructure of the nictitating gland and membrane in dog*”, was done by harvesting the nictitating membrane from both eyes of 82 dogs of different breeds, 44 males and 38 females, weighting between 2-80 kg and aged 2 months to 17 years.

The nictitating membranes were trimmed and mid-sagittaly serially sectioned, obtaining histological slides, which were stained according to the type of study, as follows: staining with hematoxylin-eosin-methylene blue (HEMB) for histological study, with periodic acid Schiff (PAS, Schiff reagent preparation following *de Tomasi* formula), Alcian blue pH 2.5, Alcian blue pH 1, PAS-Alcian blue pH 2.5 and orcein-Alcian blue pH 2.5 for histochemical study. For the natural fluorescence study were both used hematoxylin-eosin (HE) stained histological slides and exposed, undeparaffinated, unstained slides. For staining the cytological slides obtained by the nictitating gland displaying touch impression smear or the tissue scrapings, stains were used as follows: HE for cytology, May-Grunwald-Giemsa (MGG) and acid periodic Schiff-hematoxylin (PAS-H). Subsequently, the histological and cytological slides were examined at the optical microscope **MC300 Micros Austria**. The histological autofluorescence analyse of the nictitating

gland was made with the same microscope that had added an epifluorescence module with UV light, and changing the normal objectives with the ones special for fluorescence. Histological slides were examined in UV light having the wavelenght of 420-530 nm and using the filter for the green fluorescence.

Transmission electronic microscopy study was performed in transmission electron microscope **Philips CM 100**.

Nictitating gland consists of compound tubuloacinar secretory units and the interstitial tissue is formed by collagen fibers derived from the capsule, which divide the gland into glandular lobules, mioepithelial cells, plasma cells, lymphocytes and intra- and interlobular adipocytes.

The nictitating palpebral conjunctiva is thicker than the bulbar conjunctiva and is constituted by a non-keratinized stratified squamous epithelium consisting of 3 to 5 rows of cubic epithelial cells, including round and oval cells, between them being numerous goblet cells.

The bulbar nictitating conjunctiva from the proximal zone is structured by a non-keratinized stratified squamous epithelium consisting of 2 to 5 rows of prismatic, cubic, oval or flattened epithelial cells (this being also the approximate order of the disposal of the cells on the basal layer) and lacks the goblet cells.

The bulbar nictitating conjunctiva covering the lymphoid follicles, called also the lymphoid follicle-associated epithelium or lymphatic epithelium, has a wavy appearance in the mid-sagittaly histological sections of the nictitating membrane. On the lateral sides of the lymphoid follicle, the conjunctiva is reduced to a single layer of cubical cells, that at the lymphoid follicle's apex (the area closest to the epithelium) disappears from histological slides because of the fragility of the epithelial cells within (M cells).

The anterior and posterior nictitating conjunctiva epithelium from the free edge of the nictitating membrane is non-keratinized stratified squamous epithelium, comprising the generating layer, 1 to 3 polyhedral epithelial cells layers and 1 to 3 flattened superficial cells layers.

According to structural characteristics, the nictitating conjunctiva lymphoid follicles may be considered as mucosal associated lymphoid tissue (MALT) and more specifically named – *Nictitating Conjunctiva Associated Lymphoid Tissue* (NCALT). Thus, lymphoid follicles of the nictitating conjunctiva are for the first time mentioned as NCALT. Therefore, NCALT, together with CALT (*Conjunctiva-Associated Lymphoid Tissue*), LGALT (*Lacrimal Gland-Associated Lymphoid Tissue*) and LDALT (*Lacrimal Drainage-Associated Lymphoid Tissue*), are part of EALT (*Eye-Associated Lymphoid Tissue*), being part of the MALT (*Mucosa Associated Lymphoid Tissue*) at its turn.

The histochemical analysis of the results of this study show that nictitating gland of the dog present acini containing both neutral and acidic glycoproteins (sialilate and sulphate). Secretory tubules cells with wide lumen, only shows PAS-positive reaction.

In dogs, autofluorescence is distinguished by a uniform fluorescence, very weak and diffuse, sometimes more intense at the apical pole of all or some glandular epithelial cells. In cytological preparations made from touch imprint or scrape of normal nictitating gland, glandular epithelial cells appear in cytological preparations in the form of clusters of epithelial cells and/or secretory units with acidophilous cytoplasm and granular aspect, surrounding a large nucleus, dense often eccentrically located. Glandular epithelial cells were pyramidal aspect and apical pole is slightly rounded, and secretory units consist of 7-10 glandular epithelial cells.

Ultrastructural, glandular epithelial cells of the nictitating gland gland of the dog, have serous type secretion vesicles (with intense and uniform electron density) and mucosal type (with low electron density).

Chapter VI, called „*Micromorphometrical and statistical study of the nictitating gland and membrane in dog*”, was achieved using histological slides of the nictitating membrane, mid-sagittal sections, which capture the best of its all histological structures. We used histological slides stained with hematoxylin-eosin-methylene blue (HEMB) and acid periodic Shiff (PAS) and the *MC300 Micros Austria* light microscope that has attached a *Moticam 352* video camera. The micromorphometrical analysis of the histological and cytological structures was performed using the software *Motic Images Plus 2.0 ML*.

For each dog were measured the main histological structures of the nictitating gland, the main portions of the nictitating gland and various ratios established between some histological structures of the nictitating membrane. The results were statistically analyzed using **SPSS 10** software. The 82 dogs were grouped according to the zootechnic and canine systematization in breed categories: small breed dogs (under 10 kg, 24 dogs), medium breed dogs (10-25 kg, 33 dogs) and large and giant breed dogs (25-90 kg and over, 25 dogs). Within each breed category, the dogs were again grouped by age: group I (0-2 years) young dogs, group II (3-8 years) adult dogs, group III (9+ years) senior dogs. Thus, in small breed dogs, age group I is composed of seven dogs, age group II of 6 dogs and age group III of 11 dogs. In medium breed dogs, age group I included 18 dogs, group II – 9 dogs and age group III – 6 dogs. In dogs of large and giant breed, age group I included 9 dogs, group II – 8 dogs and age group III – 8 dogs. Then, within the breed category, dogs were grouped according to sex: males and females. Thus, small breed dogs were divided into 13 male dogs and 11 females, dogs of medium breed were divided into 18 males and 15 females, and large breed dogs were divided into 13 males and 12 females.

For each variable were determined descriptive statistical indicators (arithmetic average,

standard error of the average, 95% confidence interval, standard deviation, minimum and maximum value) for each age and sex group within the breed categories. We also examined whether there was a significant statistical difference between the measured histological formations averages for the age groups included into the breed categories (small, medium, large) using the *One-Way ANOVA Test* and between males and females averages within breed categories using *Independent-Sample T Test*.

From the performed study was noted that the size of the lymphoid follicles in small breed dogs from 0-2 years age group is larger (67245.82 μm^2 area/374,26 μm large diameter/206,28 μm small diameter) (p<0,01) than those from 9+ years age group (37547,61 μm^2 area/279,06 large diameter /155,8 μm small diameter). Therefore, the size of the lymphoid follicles is usually larger in young dogs (0-2 years) than in senior dogs (9+ years). It was also noted that the average thickness of the capsule of the bulbar gland is smaller (138 μm in 0-2 years and 77.4 μm in 3-8 years) than the one of the palpebral side in dogs of small breeds, age group 0-2 years (152.13 μm) and 3-8 years (109 μm), but the differences are statistically insignificant, which could be a cause of the nictitating gland protrusion.

From the study is noted that the size of lymphoid follicles of medium breed dogs, do not differ significantly between age groups (p≥0,050), but they are higher in dogs aged 0-2 years (125685.3 μm^2), followed by dogs aged 3-8 years (115977.2 μm^2) and those in the age group 9 + years (91538.35 μm^2), so the size of lymphoid follicles are usually higher in young dogs to adults and senior dogs.

It was noted that the average thickness of the capsule of the bulbar gland is smaller (120.12 μm in 0-2 years; 167.3 μm in 9+ years) than that of the palpebral side in medium breed dogs of 0-2 years age groups (130.41 μm) and 9+ years (202.6 μm), but the difference is not statistically significant (p≥0,050). However, it could be a contributing factor for the nictitating gland protrusion in young dogs of medium breeds.

Larger dimensions of the lymphoid follicles in young dogs, the thickness of the capsule of the bulbar gland, which is thinner than that on the palpebral side, and the thickness of the nictitating membrane cartilage that is thinner, can be considered contributing factors of the nictitating gland protrusion in large breed dogs, in the age group 0-2 years.

The cartilage of the nictitating membrane is thinner in the age group 0-2 years (199.4 μm) than that in the 9+ years age group (263.25 μm) of large breed dogs (p<0,001) and can favor the eversion of the nictitating membrane. In large breed dogs, the distance from the free edge of the membrane to the proximal end of the nictitating cartilage is the shortest in dogs of 0-2 years age group (1340.6 μm), followed by age groups 3-8 (1459.74 μm) and 9+ years (600.92 μm) (insignificant statistical difference). This distance, shorter in young dogs of large breeds, could

favor the eversion of the nictitating membrane, the small space between the conjunctiva and the cartilage making the adaptation more sensitive, in case of unequal development.

In all dog breeds (small, medium, large) was observed that the size of the lymphoid follicles is the largest in the 0-2 years age group (67245.82 μm^2 area – small breeds, 125685.3 μm^2 area – medium breeds, 159358.3 μm^2 area – large breeds), and decrease with age groups (37547.61 μm^2 area – small breeds, 91538.35 μm^2 area – medium breeds, 70862.92 μm^2 area – large breeds). In conclusion, the younger dogs (0-2 years), normally have larger lymphoid follicles, fact that should not be interpreted as follicular hyperplasia.

Regarding the number of the lymphoid follicles, in small and medium breed dogs their number increases with age, the age group 9+ years having the largest number of lymphoid follicles in both small (6 lymphoid follicles) and medium breeds (8.66 lymphoid follicles), but the differences do not show statistical significance. In large breed dogs, the largest number of lymphoid follicles occurs in the age group of 3-8 years (7.5 lymphoid follicles). Therefore, small and medium breed dogs, in the age group of 9+ years and large breed dogs in the age group of 3-8 years, have the largest number of lymphoid follicles, fact that should not be interpreted as follicular hyperplasia.

The interlobular connective tissue is normally thicker in females (31.22 μm) than in males (19,02 μm) ($p < 0,01$) in small breed dogs, and the intra- and interlobular ducts are larger in 3-8 years age group dogs (123.01/78,52 μm – intralobular ducts; 160.25/93,1 μm – interlobular ducts) and in females (74.25/40.29 μm – intralobular ducts; 142.29/85,75 μm – interlobular ducts) than in males (53/41.34 μm – intralobular ducts; 120.82/80.11 μm – interlobular ducts), without differences between the average to be statistically significant ($p \geq 0,050$). Therefore, the larger sizes of these histological structures in females and in 3-8 years age group of dogs from small breeds could favor the occurrence of keratoconjunctivitis *sicca*.