ABSTRACT

In rabbit, the Harderian gland is a large organ, placed in the inferior anterior-medial portion of the orbit, in direct contact with the eyeball, attached to the third eyelid. It has two well-defined lobes (a pink one - ventral and a white one - dorsal), that cover almost a half of the medial surface of the eyeball, at the profound face of the cartilaginous skeleton of the nictitating membrane and the secretion is eliminated through a proper duct, in the conjunctival sac, contributing at the production of the external lipoid fraction of the tear film.

According to the consulted references, complex histochemical studies of the Harderian gland (including the secretory products) and of the nictitating membrane in rabbit, scarcely clarified in the scientific literature, on a large number of cases, were not undertaken. Studies regarding the organogenesis, micromorphometrical evaluation of the main glandular histological structures in postnatal period of development and micromorphometry and statistics of the histological formations and secretory products of the normal Harderian gland in adult rabbit are performed for the first time, similar studies not being identified in autochthonous or foreign consulted references. Clarifying of these unknown facts is the aim of this doctoral thesis.

The first part entitled „*Literature review*" consists of two chapters that summarize the main bibliographic database of scientific literature regarding both protection and fixation annexes of the eyeball in rabbit (chapter I) and nictitating membrane, focusing on information about its profound gland, the Harderian gland (chapter II).

The second part „*Personal researches*" consists of five chapters (chapter III-VII). Chapter III presents the aim and objectives of this thesis, chapters IV, V and VI present and describe the results, including the materials and methods, discussions, interpretations, analysis and partial conclusions that are to be drawn from researches. The paper is illustrated by 186 figures, 10 tables, 2 charts and imposed consulting of 320 reference titles. In chapter VII, the final conclusions are presented and synthesize the conducted researches.

Investigations in chapter IV entitled ,,*Organogenesis of the Harderian gland and the nictitating membrane in rabbit*" were realized by harvesting of the histological pieces from 67 rabbit fetuses, respectively from 11 does: 23 fetuses 16 days old (from three does: 6, 8 and 9 fetuses), 13 fetuses 20 days old (from two does: 6 and 7 fetuses), 9 fetuses 24 days old (from two does: 4 and 5 fetuses) and 22 fetuses 20 days old (from four does: 5, 5, 6 and 6 fetuses). The embryonic development was although followed by echographic exam, in order to be taken appropriate measures in case of nonpregnant females, protection of pregnant and removal of the sterile does from the lot.

From the harvested histological pieces (in 16 and 20 days old fetuses the entire head was embedded in wax and in 24 and 28 days old fetuses the nictitating membrane along with the Harderian gland were highlighted through medio-sagittal sectioning of the cranium, isolating the orbit with its content) serially sections were realized and histological slides obtained through study specific staining techniques, as follows: for morphological details, the histological slides were stained haematoxylin-eosin (HE) and haematoxylin-eosin-methylene blue (HEMB) and for various histochemical aspects: periodic acid Schiff (PAS) and Novelli.

The nictitating membrane organogenesis in rabbit is initiated in the 16^{th} gestational day as a mesenchymal fold covered by conjunctiva, that constitutes the primordial nictitating membrane with 87,89 µm in length.

The conjunctiv-mesenchymal fold is elongated and narrowed in 20 days old rabbit fetuses, and the beginning of the cartilaginous histogenesis is observed within its proximal thickness as a mesenchymal cell agglomeration under the form of a band along the longitudinal axis of the primordial nictitating membrane.

In 24 days old rabbit fetuses, the nictitating membrane cartilage has all the morphological structures as adult animals and in 28 days old rabbit fetuses consists of chondrocytes in chondroplasts, completely covered by cartilaginous matrix material and the outline is covered by chondroblasts and fibroblasts that structure the perichondrium.

Organogenesis of the profound gland of the third eyelid is already initiated is 20 days old rabbit fetuses. At the future nictitating membrane base is highlighted the presence of an irregular cellular mass, structured by epithelial cords, non-patent and with few, small ramifications.

In 24 days old rabbit fetuses organization of the Harderian gland is highlighted at the base of the nictitating membrane cartilage by primordial epithelial cords (non-patent) and primordial ducts (patent) forming, a few of those having ramifications with alveolar formations differentiating in the organogenesis process. The principal primordial ducts (patent) have secondary tubular ramifications at whose ends alveolar units form and detach.

The cellular cords, primordial glandular ducts and alveolar units number increase and start to associate in order to form glandular lobules. The primordial alveolar formations, unlike the acinos celular mass, present the beginning of a central lumen formation and are structured by one or two rows of cuboidal and concentrically arranged glandular epithelial cells. Their dimensions, in 28 days old rabbit fetuses, do not register a significant growth, but their density in the fetal glandular tissue show considerable increase.

In prenatal period, the Harderian gland does not show cytological and cytochemical characters in order to initiate the specific lipoid secretion, making possible to conclude that is still a immature structure in newborn rabbits, following that functional maturation to realize in the postnatal period.

The researches described in chapter V, entitled „*Postnatal development of the Harderian gland and nictitating membrane in rabbit*", were realized by harvesting a number of 60 Harderian glands attached to the nictitating membrane from 30 rabbits, three for each age category: 1, 2, 3, 5, 7, 10, 14, 21, 28 and 180 days old (adult).

The histological study of tracking the development process of the Harderian gland in rabbit during the postnatal period, in order to establish the start of secretory activity and to observe its differencing mechanism, was realized by performing permanent histological slides of samples harvested from rabbits, grouped in 10 age categories. In order to observe the morphological details, HE and HEMB stains were applied and histochemical stains: PAS (neutral glycoprotein) and counterstaining with HE and Neutral red, after post fixation of the samples in osmium tetraoxide, in order to highlight the lipid secretory product of the glandular epithelial cells.

Micromorphometrical and statistical study was realized on images obtained from permanent histological slides, using the light *Micros Austria MC300* microscope with *Moticam 352* video camera attached. The micromorphometrical analysis was realized using the *Motic Images Plus 2.0 ML soft.*

The areas of glandular epithelial cells of the two lobes and of their nuclei were measured, in the statistical analysis the histological formations being named variables. Per each case, 50 measurements were performed, respectively 150 measurements for each variable, in each age category. The results were statistically processed using the *SPSS 17.0* statistical program. The 30 rabbits were grouped in 10 age categories, three in each group.

For each variable were determined descriptive statistical indicators (arithmetic mean, standard error of the mean, 95% confidence interval, standard deviation, minimum and maximum value), for each age category, also chronological series of moments were performed for variable evolution describing.

In newborn rabbits, the Harderian gland has the same topography and size in relation to the surrounding structures from the orbit, as in adult animals. At microscopically examination though, the gland presents the morphological characteristics of an immature structure, abundant connective fibers being present between alveolar units, also the two glandular epithelial cells have not yet been differentiated.

It is structured by ramified secretory tubules, with narrow lumen, separated by delicate connective fibers. The future secretory cells have various forms, some being cuboidal, others low columnar, the glandular epithelium appearing sometimes stratified. An important aspect at this age is represented by the nucleus/cell surface ratio, which is 1/3.

In the third day after calving, the most important morphological characteristic of the epithelial tissue is represented by highlighting of the lipid droplets, in the slides obtained by post fixation with osmium tetraoxide, in the cytoplasm of the glandular epithelial cells that structure the pink lobe. Their presence represents the proof of the gland secretory activity debut, even though it is still an immature structure, because in the glandular cells from the white lobe are not yet visible the lipid droplets, through light microscopy.

In the seventh day of neonatal development, the two lobes of the Harderian gland in rabbit can be differentiated macroscopically and characterised based on their colour as the pink lobe and the white lobe, as the gland in adult animals.

Major changes are evidentiated in glandular morphology, compared to its development status observed in the first day after calving, a pronounced increase of alveolar diameters standing out. The two types of glandular epithelial cells have completed their ultrastructure, by lipid droplets in cytoplasm of the epithelial cells from the white lobe becoming visible in light microscopy, coloured in grey-dark grey after post fixation of the samples in osmium tetraoxide, because of their saturated lipid content.

In 2 weeks old rabbits, the postnatal development of the Harderian gland appears to be complete, further changes occurring mainly in the interstitial space. All secretory cells have typical cytological characteristics for an intense activity of lipids secretion.

In the secretory tubules lumen can sometimes be observed agglomerations of lipid droplets, with the same diameter as intracytoplasmic ones. This image is characteristic to the pink lobe. In some alveolar lumens, within the white lobe, agglomerations of pigmentary material are visible, most probably representing the debut of porphyrin secretion.

In 21 days old rabbits, the Harderian gland presents its morphological characteristic as adult animals. The glandular territory is well divided in lobules through delicate connective tissue, detached from the capsule and intra- and interlobular blood vessels, more numerous within the pink lobe, provide the cellular nutrients.

In addition to the pink and white lobes, a mixed portion is highlighted at the delineation area between the two, on glandular alveolar structure basis. The morphological characteristic of the mixed area is represented by the presence of both epithelial cells characteristic to the white lobe (with small intracytoplasmic lipid droplets) and epithelial cells characteristic to the pink lobe (with large intracytoplasmic lipid droplets) in the structure of one alveolus. This aspect becomes visible once with the morphological differentiation of the epithelial cells from the white lobe, around the seventh day of postnatal development, following the appearance of the intracytoplasmic lipid droplets with small diameter, in the epithelial cells from the white lobe.

In the fourth week, the Harderian gland presents all the morphological and functional characteristics as that in adult animals.

The glandular alveoli within the white lobe consist of columnar epithelial cells with round nuclei, of 26,57 μm^2 average surface, placed in the lower third. The nucleus/cell surface ratio has a value of 1/7, very close to that in adult animals.

The glandular alveoli within the pink lobe consist of columnar epithelial cells with large nuclei, of 29,4 μm^2 average surface, placed in the lower third. The nucleus/cell surface ratio has a value of 1/7, close to that in adult animals.

In the **white lobe**, *the growth rhythm of average cellular area* compared to the first day is of 44.16% in the 2^{nd} day, in the 3^{rd} day is of 58.37%, in the 5^{th} day is of 85,09%, in the 7^{th} day is of 87.55%, in the 10^{th} day is of 108.15%, in the 14^{th} day is of 148.22%, in the 21^{st} day is of 224.99%, in the 28^{th} day is of 275.25%, and in 180^{th} day (in adult) is of 342.88%.

In the **pink lobe**, *the growth rhythm of average cellular area* compared to the first day is of 0.70% in the 2^{nd} day, in the 3^{rd} day is of 85.46%, in the 5^{th} day is of 114.52%, in the 7^{th} day is of 118.59%, in the 10^{th} day is of 155.68%, in the 14^{th} day is of 197.35%, in the 21^{st} day is of 281.78%, in the 28^{th} day is of 333.60%, and in 180^{th} day (in adult) is of 537.52%.

Chapter VI, entitled „ *Histology, histochemistry, ultrastructure and micromorphometry of the Harderian gland and nictitating membrane in rabbit*" was realized by harvesting the Harderian glands and the nictitating membranes from 30 adult rabbits, from private breeders.

The study concerning the histological structure and histochemical aspects of the Harderian gland and nictitating membrane in rabbit was also realized by performing permanent histological slides of harvested samples, stained HE and HEMB in order to observe the morphological details and histochemical stains: PAS (neutral glycoproteins), PAS-alcian Blue pH 2,5 (acid and neutral glycoproteins), Van Gieson, Masson Trichromic, Sudan IV (neutral saturated lipids), Oil red O (neutral saturated lipids), Nile Blue (fatty acids, neutral saturated lipids and acid lipids), counterstained with HE and Neutral red, after post fixation of the samples in osmium tetraoxide (saturated and unsaturated lipids) and stained Sudan IV after post fixation

in potassium dichromate (unsaturated lipids), in order to establish the chemical composition of different histological structures of the nictitating membrane and of the Harderian gland secretory product.

The transmission electron microscopy study was realized on ultrathin sections of adult rabbit Harderian gland, the images being obtained using the *Tesla BS 500* and *Phillips CM 100* transmission electron microscopes.

For the micromorphometrical and statistical study were measured the alveolar and tubular areas from both lobes, the areas of glandular epithelial cells and of their nuclei from the two lobes, lipid droplets diameters and porphyrin deposits areas from both lobes. For each variable 50 measurements were performed, on 10 cases, respectively 300 measurements for each variable and were determined descriptive statistical indicators (arithmetic mean, standard error of the mean, 95% confidence interval, standard deviation, minimum and maximum value).

At the histological examination, the nictitating membrane appears to be formed by a conjunctival fold, containing an oval shaped portion of cartilage. This has a saucer form with its concavity molded on the eyeball's convexity and it is of hyaline type, the rest of the nictitating membrane consisting of glandular structures, adipose tissue and connective tissue.

The Harderian gland consists of compound tubuloalveolar secretory units, which organize as lobules, separated by connective tissue septa, derived from the capsule. Between the alveoli and the tubules, blood vessels and intralobular ducts can be observed. The tubuloalveolar units are surrounded by myoepithelial cells, and the interstitial space contains plasmocytes, lymphocytes and interlobular ducts.

The columnar epithelial cells of the white lobe alveoli have an average value of the area of 215,64 μm^2, with round nuclei, placed in the lower third, with evident nucleoli and the cytoplasm is vacuolated. The nucleus/cell surface ratio has a value of 1/8 in adult rabbits.

The glandular alveoli from the pink lobe consist of columnar epithelial cells with an average area of 310,41 μm^2, with the nuclei placed in the lower third and the cytoplasm is highly vacuolated. The nucleus/cell surface ratio has a value of 1/9.

The histochemical stains highlight the presence of immunocompetent cells (plasmocytes) in the interstitial space of the Harderian gland, beside of the common components, more numerous in the white lobe than in the pink one, and we can conclude that the white lobe has a more important role in ensuring the local immune response.

The positive reaction to both stains, PAS and Alcian blue, of the hyaline cartilage of the nictitating membrane, through a mixture of colours magenta-blue, reveals the presence of neutral and acid glycoproteins in its chemical composition.

The acinar glandular structures that cover a large portion of the palpebral surface of the third eyelid cartilage are of mixed type (serous and mucous) and the secretion is completed by the mixed one from the excretory ducts, fact demonstrated by their positive reaction for neutral and acid glycoproteins.

The Harderian gland in rabbit presents a very scarce secretion of both types of glycoproteins from the tubuloalveolar units, but these are elaborated in the cells of the excretory glandular ducts and added at the typical lipidic secretion of the gland.

Porphyrins are highlighted on the territory of the both lobes as intraluminal agglomerations of pigmentar material, with various forms and dimensions, hyaloid aspect and having tinctorial affinity for most of the used staining agents that demonstrates a complex chemical composition.

The lipids secreted by the epithelial cells of the alveoli and tubules of the Harderian gland in rabbits are saturated (negative reaction in postcromation - Sudan IV; osmium tetraoxide - weak positive), predominantly neutral lipids (Sudan IV - intense positive reaction, Oil Red O - intense positive, Nile blue sulphuric solution - intense positive), but there are present even the acid ones, in small proportion (Nile blue sulphuric solution - positive).

The study of ultrastructure highlights the spot desmosomes junctions between the columnar cells of the glandular alveoli from both lobes, to which revealing the adjacent plasma membranes are participating at a distance of 30-35 nm.

The glandular cells cytoplasm contains vesicles with diameters of 1400-1800 nm in the white lobe and with larger diameters - 4000-5000 nm, in the pink lobe, that present very low electronic density and are covered by endomembranes. The close contacts between the smooth endoplasmic reticulum, the lipid droplets and the extern membrane of the mitochondria, demonstrates the cooperation in order to realize the cell metabolic processes for an increase secretion of lipids.

At the inner surface of the endomembranes of the secretory product was observed a dark coloured precipitate, interpreted as a chemical compound resulted after the reaction between lipids and osmium tetraoxide.

Micromorphometrical analysis and descriptive statistic of the main morfofunctional units and of the main secretory products of both lobes of the Harderian gland in adult rabbit, led to establishment of the normal dimensions of this formations and offer reference values for following eventual experimental studies regarding the effect of various environmental or pathogen factors on the morphology and secretory activity of the gland.

The average areas of the glandular alveoli and tubules are of 9470,68 μm^2 in the white lobe and of 13735,67 μm^2 in the pink lobe.

The average diameter of the lipid droplets is of 1,60 µm in epithelial cells within the white lobe and of 4,60 µm in cells within the pink lobe.

The average area of the porphyrin deposits is of 1807,03 μm^2 in the alveolar lumen of the white lobe and of 3317,66 μm^2 in the alveolar lumen of the pink lobe.