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RESEARCH ARTICLE

Morphology of the Lacrimal Gland and Superficial Gland of the Third Eyelid of Roe Deer (*Capreolus Capreolus L.*)

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ABSTRACT

This study on the lacrimal gland (LG) and the superficial gland of the third eyelid (SGTE) was conducted on 21 sexually mature Roe deer (11 males and 10 females). The research material was obtained from Roe deer shot by the Hunting Association during their prescribed hunting season. The shape and topography of the glands are described macroscopically. The LG has a triangular shape in all the investigated individuals and it is located in the dorsolateral angle of the orbit, between the tendons of the dorsal rectus and the lateral rectus muscles of the eyeball. The SGTE is oval in shape and it is located between the medial rectus muscle and the ventral rectus muscle of the eyeball and it is partially covered with the ventral oblique muscle of the eyeball. The SGTE is situated around the cartilage of the third eyelid During histological and ultrastructural analyses using light and transmission electron microscopy, it was established that the LG is a tubulo-acinar gland. Histochemical examination demonstrates that excretory cells are in general PAS-negative and Alcian blue pH 2.5-negative, and thus this gland can be regarded as a serous gland. The SGTE exhibits also a tubulo-acinar morphology. The histochemical study showed that the SGTE is of a combined nature, i.e. with serous cells (PAS-negative and Alcian blue pH 2.5-negative), mucous (PAS-positive and Alcian blue pH 2.5-positive) and seromucous cells (PAS-positive and Alcian blue pH 2.5-positive). Upon electron microscopic examination, LG and SGTE secretory cells exhibited a similar ultrastructure appearance, with secretory cells tightly filled with intracytoplasmatic secretory granules. Neither the body size nor gender had a significant influence on the size of the LG and the SGTE (t-test: $P>0.05$). The ultrastructure and function of the investigated eyeball's glands in Roe deer was similar to those observed in other species, like cattle, camel and bison.

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INTRODUCTION

The LG and the SGTE are accessory organs of the eye. The role of these glands is to secrete fluids - serous, mucous or seromucous as part of one of the three layers of tear film. The first layer is water layer produced by the LG, accessory (Krause's) lacrimal glands situated in the fornix conjunctive superior and fornix conjunctive inferior and accessory (Wolfring's) lacrimal glands situated above the superior and inferior edge of the tarsal cartilage (Jordan, 1990). The second layer is a fatty layer produced by the tarsal (Meibom's) and sebaceous (Zeis') and ciliary (Moll's) glands. The third layer is a mucin layer produced by conjunctival goblet cells known as Manz's glands (clusters of cells situated in the conjunctival part of the corneal limbus), Henl's crypts (the posterior surface of the superior eyelid, slightly below the superior edge of the tarsal cartilage) and the SGTE (Jordan, 1990). The tear

film consists of water, electrolytes and proteins (Dartt, 2009; Funaki *et al*., 2010) and has several functions, e.g. it prevents the cornea and the conjunctiva from drying up, contributes to metabolite exchange, nourishes and lubricates of the eye, protects the cornea surface from damage caused by foreign bodies and has bactericidal and bacteriostatic properties (Zagon *et al*., 2012). The LG secretory function and the tear film composition (eg. lactoferrin) influences on the eye physiology and pathology (Flanagan and Willcox, 2009; Kawashima *et al*., 2012).

The proper composition of the tear film is thus of principal significance for normal eye physiology. The role of the third eyelid is mechanical protection of the cornea, as well as local immunological protection by substances present in lymphoid nodules and tear film distribution on the cornea surface. The movement of the third eyelid in animals is of a passive character which is connected with the lack of muscles actively moving the eyelid. The proper position of a free edge of the third eyelid is controlled by the position of the eyeball. Retraction of the eyeball as a result of the retractor bulbi oculi muscle contraction causes protrusion of the third eyelid towards the temporal angle of the eyeball (Lavach, 1990).

The LG and SGTE in pigs, horses, guinea pigs, rabbits and primates are tubulo-acinar structures that produce a secretion of mucoalbuminous character (Klećkowska-Nawrot and Dzięgiel, 2007, 2008; Ding *et al*., 2010; Schechter *et al*., 2010). According to Gargiulo *et al*. (1999) and Mohammadpour (2011), the LG in sheep and camel have been considered by most investigators to be mixed glands consisting of both serous and mucous alveoli, with the former more numerous at the periphery of the glandular lobules. Pinard *et al*. (2003) proved the double-lobar structure of the LG in cattle. Similar anatomical build, with the exception of an accessory lobe occurred in camel. The bilateral asymmetry of the mentioned LG was stated in humped camel (mean length and width of the LG of the left side was bigger than that of the right side and the differences were significant ($P<0.05$).This LG was elongated and irregular in outline. It was located in the dorsolateral angle the orbit, in the neighborhood of the frontal bone zygomatic process (Mohammadpour, 2008).

The aim of this study was to describe the normal anatomical, histological, histochemical and ultrastructural findings in Roe deer LGs and SGTEs. Secondly, comparison of the obtained results was made on analogical glands in other domestic ruminants (cattle, sheep, goat) on the basis of the available literature. Functional and anatomical differences in the glands under study between domestic and wild-living ruminants have been described in this paper.

MATERIALS AND METHODS

The study was conducted on 21 Roe deer (11 males and 10 females), with a BW of between 20 to 25 kg, and height at withers from 69 to 72 cm. The animal age was estimated as adult on the basis of dentition. The research material came from seasonal animal shooting by the Hunting Association.

Macroscopic evaluation: Morphometric measurements of glands were conducted using an electronic slide caliper with an accuracy of 0.1 mm. Normality of frequency distribution of the analyzed variables (body side and sex) was verified with the use of the Shapiro-Wilk test. The significance of the differences between average values of the compared variables was estimated with the Student's t-test. The test was applied to assess the statistical significance of the observed differences. All the analyses were performed with the use of Statistica 7.1.

The shape of the analyzed glands and their topography (holotopy-location of an organ in the body and syntopy-location of some organs in relation to each other) were examined in the study.

Histological analysis: The research material was directly fixed in 4% buffered formaldehyde, rinsed in running water for 24 h, dehydrated in an alcohol series of increasing concentration (75% for 20 hrs, 96% for 8 hrs, 99.8% for 12 hrs), embedded in paraffin and cut on Leica RM 2045 rotation microtome (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) into 7μ_sections. The samples were stained with hematoxylin-eosin (H-E) method and examined using an Olympus BX 41 (Olympus, Tokyo, Japan) light microscope for histological description.

Histochemical analysis: The histochemical analysis including periodic acid-Schiff (PAS) and Alcian blue pH 2.5 staining was conducted in order to identify the presence of glycoproteins and muco-substances. Alcian blue pH 2.5 and PAS staining scoring system was based on standard protocol (Spicer and Henson, 1967) in Electron Microscopy Laboratory at Wroclaw University of Environmental and Life Sciences.

Transmission electron microscopic analysis: The collected material (LG and SGTE) was fixed in 2.5% glutaraldehyde on a 0.1 M phosphate buffer of pH 7.4, and was rinsed in a phosphate buffer. The material was then postfixed in 4% OsO4 for 2 h. After re-rinsing in a phosphate buffer, the fragments of the glands were dehydrated in an acetone series (from 30 to 100%). The dehydrated material was immersed in Epon 812 epoxide resin. The blocks were cut into 70 nm pieces using an MTX ultramicrotome (Leica Microsystem Wetzlar GmbH, Wetzlar, Germany). The preparations were observed through a Tesla BS 500 transmission electron microscope.

RESULTS

Gross examination: The LG was situated in the dorsolateral angle of the orbit between the tendons of the dorsal rectus and the lateral rectus muscles. It was triangular in shape with its base oriented towards the fornix conjunctivae superior and its apex oriented caudally towards the tip of the periorbital conus. In gross appearance, it was a uniform, undivided gland. The mean size (length x width x thickness with SD) of the LG was 27.6 (±4.6) x 13.0 (±2.0) x 2.2 (±0.7) mm in females and 26.4 (±4.4) x 12.6 (±2.2) x 1.8 (±0.4) mm in males, respectively. The differences were not statistically significant (t-test: $P>0.3$, $P>0.4$, $P>0.05$, respectively for three dimensions) and no correlation was observed between the body size and the size of the LG (t-test: $P>0.5$, $P>0.9$, $P>0.2$, respectively) (Table 1).

The SGTE was situated around the cartilage of the third eyelid. It is located between the medial rectus muscle, the ventral rectus muscle and is partially covered with the ventral oblique muscle of the eyeball. The SGTE is oval in shape. It adjoins the frontal edge of the deep gland of the third eyelid. The third eyelid is made of cartilage that is composed of a superior and inferior arm and the so-called crosspiece. The superior and inferior arms stiffen the fold of the lid, while the crosspiece is oriented towards the tip of the periorbital conus. The third eyelid crosspiece is surrounded by the SGTE. The mean of the superficial gland in females and males showed statistically insignificant differences (t-test: P>0.3, P>0.4, P>0.05, respectively for three dimensions). Similarly, no correlation occurred between the body side and the size of the superficial gland (t-test: P>0.5, P>0.9, P>0.2, respectively) (Table 1).

Histological examination: The LG in Roe deer is a tubulo-acinar gland with lobules surrounded by relatively poor connective tissue. The acini are composed of 2 types of cells, i.e. tall conical cells surrounded by basal, myoepithelial cells, where the former encompass a small lumen (serous cells) and sporadic conical cells with a large and irregular lumen (mucous cells). The secretory cell nuclei are round to oval and are located both in the basal areas and central parts of the cells. These cells have basophilic granular and vacuolated cytoplasm. The glandular secretion is aggregated in the apical parts of the cells. The nuclei of the mucus-producing cells are oval and they concentrate near the base of the cell. The excretory ducts are lined with a basal layer of cuboidal cells with nuclei located in the central part of the cell.

The SGTE is a tubulo-acinar gland of a mucoproteinous character with a predominance of serous secretory segments (acini of the tall conical cells with a small lumen) surrounded by typical stromal tissue. Additionally, crescent-shaped patches of mucous tubercules with the base composed of the acini of serous cells have been observed in seromucous gland (Gianuzzi's crescents). The serous cells have basophilic granular, vacuolated cytoplasm and rounded nuclei located in their basal part. The mucous cells have eosinophilic granular, uniform cytoplasm and a large irregular lumen. The nuclei of the mucus-producing cells are oval in shape and located close to the base of the cells. The excretory ducts are lined with the basal layer of cubical cells with nuclei located in the central part of the cell.

Histochemical analysis: PAS staining of the LGs studied demonstrated the presence of a few secretory cells containing slightly PAS positive granules (+) (2-3 mucous cells) while the remaining ones were serous i.e. PAS negative. Apical parts of the excretory ducts also demonstrated a slightly positive PAS reaction (Fig. 1). Alcian blue pH 2.5 staining demonstrated the presence of moderately negative granules in apical parts of the secretory cells and the epithelial cells in the interlobular ducts. We also observed the presence of single cells fully filled with Alcian blue pH 2.5 positive granules (+) (Fig. 2).

The staining of the SGTE with the PAS method demonstrated a considerable predominance of secretory segments of a serous nature which were PAS negative, with scattered PAS positive cells (++) of a mucous nature. There were also some seromucous cells present-PAS positive cells (+). Furthermore, a considerable number of detached cells in the interlobular ducts were observed which might indicate an intensified secretion of the glands under analysis (Fig. 3). The staining with Alcian blue pH 2.5 method demonstrated the presence of positive granules in seromucosal cells (+/++) and mucosal cells (++/+++). The majority of cells were serous cells Alcian blue pH 2.5 negative (-) (Fig. 4).

Electron microscopic analysis: Upon electron microscopic (TEM) examination, the secretory cells of the LG and SGTE exhibited a similar ultrastructural appearance. The analyzed cells showed the presence of large irregularly shaped or round nuclei. The majority of these cells were tightly filled with intracytoplasmic secretory granules. However, some cells especially of the superficial gland were only partially filled with their granules. These secretory granules within individual cells had well defined borders and were mostly of moderate to high electron density (Fig. 5). In general, the most electron-dense granules were situated in the peripheral regions of each cell. Apart from these afore-mentioned cells with granules of moderate to high density, some other cells were also observed which exhibited the presence of larger granules of a much lower electron density (Fig. 6). Part of those granules was fused forming bigger irregular electron-light areas. The character of the granules content was mainly homogenous but sometimes also slightly granular especially inside the granules of lower electron density. Some analyzed cells showed a particularly highly developed rough endoplasmatic reticulum (RER) in the form of structures resembling flattened cisterns.

Table 1: Lacrimal gland and superficial gland of the third eyelid measurements in Roe deer

Measurement (mm)	Sex	N	Average	Confidence interval -95%	Confidence interval +95%	Minimum	Maximum
Lacrimal gland							
Length	F	20	27.6	25.5	29.8	20.7	34.4
Width	F	20	13.0	12.1	13.9	8.5	16.4
Thickness	F	20	2.2	1.9	2.5	0.9	3.7
Length	M	22	26.4	24.5	28.4	18.3	34.9
Width	M	22	12.6	11.6	13.5	8.0	16.9
Thickness	M	22	1.8	1.6	2.0	1.3	3.1
Superficial gland							
Length	F	20	13.1	12.2	14.0	10.2	17.0
Width	F	20	10.4	9.4	11.4	7.7	15.8
Thickness	F	20	2.1	1.7	2.4	1.0	3.7
Length	M	22	12.7	12.1	13.4	10.8	15.6
Width	M	22	9.5	8.6	10.3	5.2	11.8
Thickness	M	22	2.4	2.1	2.6	1.4	3.7

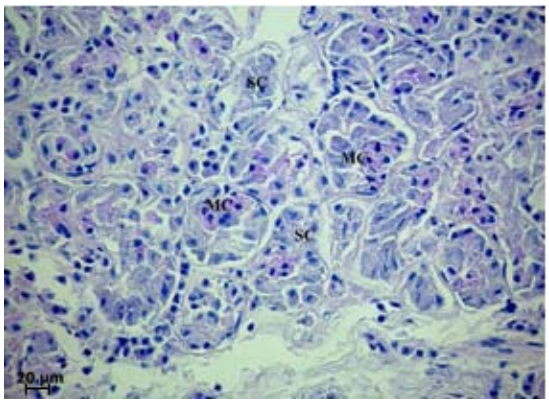

Fig. 1: Section of the lacrimal gland of Roe deer, PAS. Visible different PAS staining of mucous cells (MC) – PAS (+) and serous cells (SC) – PAS (-). Bar = 20 µm.

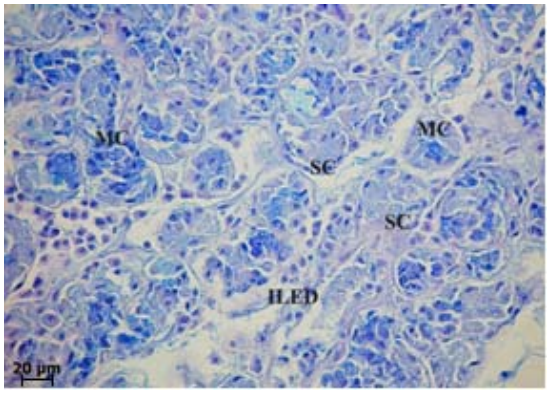

Fig. 2: Section of the lacrimal gland of Roe deer, Alcian blue pH 2.5. Note Alcian blue pH 2.5 staining of serous cells (SC) – Alcian blue pH 2.5 (-) and mucous cells (MC) -- Alcian blue pH 2.5 (+). ILED – interlobular excretory duct. Bar = 20 µm.

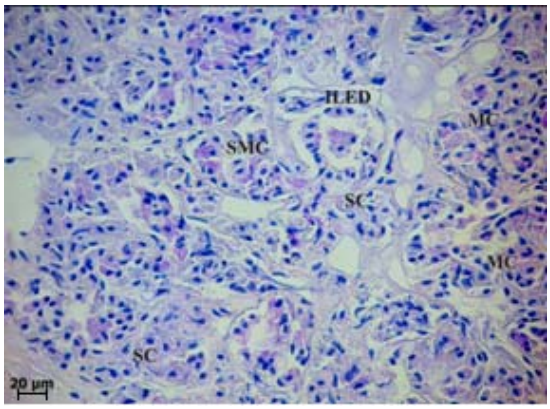

Fig. 3: Section of the superficial gland of the third eyelid of Roe deer. Note PAS staining of seromucous cells (SMC) – PAS (+), serous cells (SC) – PAS (-) and mucous cells (MC) – PAS (++/+++). The interlobular excretory duct (ILED) was filled with PAS positive secret. Bar = 20 µm.

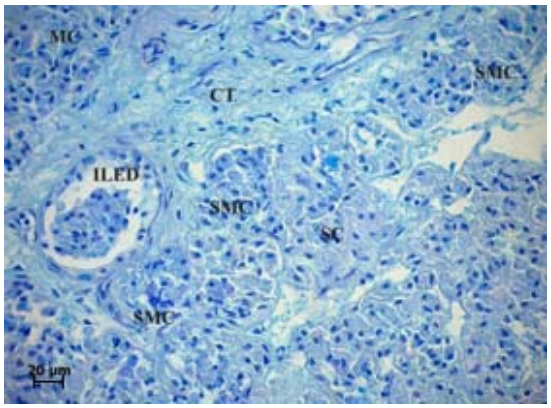

Fig. 4: Section of the superficial gland of the third eyelid of Roe deer, Alcian blue pH 2.5. Visible different Alcian blue pH 2.5 staining of serous cells (SC) – Alcian blue pH 2.5 (-), mucous cells (MC) -- Alcian blue pH 2.5 (++/+++) and seromucous cells (SMC) – Alcian blue pH 2.5 (+/++). ILED-interlobular excretory duct, CT – connective tissue. Bar = 20 µm.

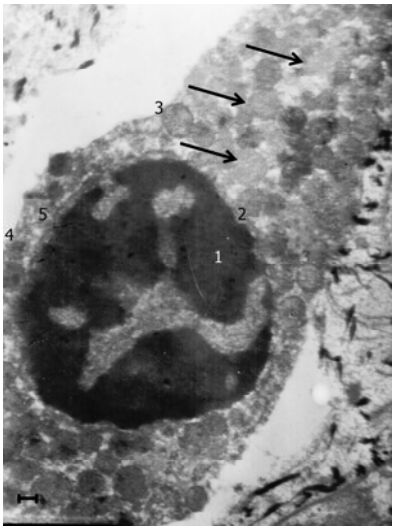

Fig. 5: Electron micrograph of the lacrimal gland cell of Roe deer. Numerous oval to round granules with contents of moderately to high electron density can be seen in the cytoplasm; part of the granules are filled with a less electron-dense homogenous material (solid arrows). 1-nucleus, 2-capsule of the nucleus and perinuclear space, 3-mitochondrion, 4-vesicles of Golgi apparatus, 5 – primary lysosome. Bar = 0.1 µm

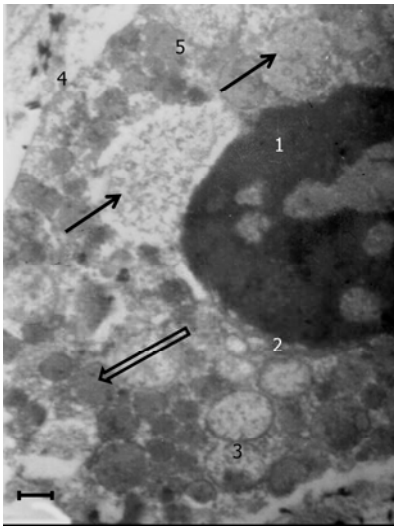

Fig. 6: Electron micrograph of the superficial gland cell of Roe deer; note the presence of intracytoplasmic secretory granules of different electron densities; majority of the granules exhibits moderately to high electron density (open arrow), while some are bigger, more electron-lucent with slightly granular contents (black arrow). 1-nucleus, 2- capsule of the nucleus and perinuclear space, 3-mitochondrion, 4- vesicles of Golgi apparatus, 5-primary lysosome. Bar = 0.6 µm.

DISCUSSION

No influence of sex on differences in anatomical and histological structure of the LG and the SGTE in Roe deer was observed in the present study. Such differences were, however, noted in the LGs of rats, mice, guinea pigs and humans. Van Haeringen (1997) demonstrated that acinar lumens were larger in males than in females. Pinard *et al*. (2003) observed that in the case of bison, age might not determine the differences in the anatomical and histological structure of the LGs, but the animal age might influence the lacrimal system proteins secretion in humans and rats (Draper *et al*., 1998; 1999).

Our study demonstrated that the LGs in Roe deer were macroscopically uniform in structure, where no secondary parts can be distinguished. Neither the side of the body nor sex influenced the size of the LG and the SGTE. Statistical analysis proved that dimensions of both glands lack significance in Roe deer. Similar results were found in the morphometric investigations of bison (Pinard *et al*., 2003). Moreover, Mohammadpour (2008) observed the lack of an accessory lacrimal gland in camels, which is typical part in cattle LG. In our study, the LGs of Roe deer were much smaller compared to those reported in cattle and camels, and their structure was of a tubulo-acinar serous type. A similar tubulo-acinar structure was found, but with a combined secretory type, in cattle and in sheep (Gargiulo *et al*., 1999). The LG in American bison (*Bison bison*) contains acini with basophilic granules, in turn cattle acinar cells have eosinophilic granules and a uniform cytoplasm. No appreciable differences were seen between cattle and bison in the histochemical study (PAS and Alcian blue) (Pinard *et al*., 2003). Pinard *et al*. (2003) further stated that the acini contain PAS-positive granules in LGs in cattle and bison within the mixed mucoserous gland cells and the tubules are serous. In our study, the LG acini were composed of a single layer of tall conical cells encompassing a small lumen producing a secretion of a serous nature. The cytoplasm in the serous cells contained basophilic granules, and PAS staining demonstrated the majority of PAS negative granules. Alcian blue pH 2.5 staining indicated the presence of moderate negative granules in apical parts of the acini cells and excretory ducts epithelium. Moreover, Alcian blue pH 2.5 staining process revealed the presence of single cells fully filled with positive granules. These aforementioned staining differences suggest a different nature of LG secretion in Roe deer (serous) and other species (serous or mucoserous).

The cartilage of the third eyelid was surrounded by the proximal part of the SGTE in Roe deer. This gland had a lobulated or cobblestoned appearance. In the bison samples, this gland extended significantly caudally from the distal edge of the cartilage as compared with cattle (Pinard *et al*., 2003). The SGTE in Roe deer was a tubulo-acinar gland with basophilic granules in cytoplasm. Similar observations have been reported in bison (Pinard *et al*., 2003), whereas in cattle they reported that the SGTE gland cells were rich in eosinophilic granules. PAS staining of SGTEs proved minority of PAS positive scattered cells (mucous) and majority of PAS negative secretory segments (serous) in Roe deer. Moreover, there were also seromucous cells within the gland tissue. Pinard *et al*. (2003) demonstrated the presence of positive granules in all acini in PAS and Alcian blue examinations of the SGTE in cattle and bison. The SGTE is a tubulo-acinar gland producing a secretion of a serous or mucoproteinous nature in cattle, similarly as camel and bison (Mohammadpour, 2008). These studies suggested that the SGTE in Roe deer and other species may potentially be of a combined secretory type.

During TEM investigations, similar ultrastructural appearance of the LG and SGTE in Roe deer was stated. The majority of cells contained tightly packed intracytoplasmic secretory granules, but some cells of the superficial gland were only partially filled with the above-mentioned granules. The study by Gargiulo *et al*. (1999) in TEM on the LG in sheep demonstrated that mucous cells have a rough endoplasmic reticulum that is reduced to a few cisterns located near the base of cells and along excretory droplets. Serous cells were characterized by the presence either of uniformly electron-dense granules or composed of dense inclusions dispersed in an electron-lucent matrix. In our study, two kinds of electron density of granules were observed (moderate to high and large, but low density granules). The predominantly serous nature of the investigated LG in Roe deer suggests a strong development of rough endoplasmatic reticulum (RER) together with excretory granule density. The study by Gargiulo *et al*. (2000) indicated that the LG in sheep produces a serous, mucoserous and mucous secretion components and that an excellent correlation existed between the secretory granule substructure and glycoprotein localization.

Conclusion: Our investigation showed a typical serous nature of the LG and combined secretory type (predominantly serous forming) in the SGTE in Roe deer. The fractions of tear film produced by the analyzed glands have both a serous (water layer) and mucous component (mucin layer). Together with the fatty layer (not examined by us), which is the fluid component of the tear film, in Roe deer the film tear presents the classic pattern of this secretion, which allows for the physiological functions of tear film maintenance (Lavach, 1990).

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