STRUCTURAL FEATURES OF THE CORNEA: LIGHT AND ELECTRON MICROSCOPY

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Summary. The aim of this study was to examine and compare the cornea structure of young and adult dogs, and pigs using light and electron microscopy. Young (10-15-day-old) and adult (over 8 month) dogs, and young (2-day-old) and adult (8-9 month) pigs were used in this study. We used 4 eyes of young dogs, 12 eyes of adult dogs, 12 eyes of piglets and 12 eyes of adult pigs. Corneal thickness was measured using ultrasonic pachymeter. Later cornea was taken for light (from young and adult pigs, and only from adult dogs) and transmission electron microscopy.

Ultrasonic measurements of cornea thickness showed that young and adult pig cornea was significantly thicker than cornea of young and adult dogs. Cornea of adult individuals was thinner than that of young ones.

Light microscopy showed the Descemet's membrane seen clearly in adult pigs and dogs' cornea but in piglets' cornea did the definite layer not mould.

Electron microscopy of the young and adult dogs revealed that corneal epithelium contained several layers of the cells. The basement membrane of the corneal epithelium cells was clearly seen in both young and adult dogs cornea. Superficial cells of the cornea epithelium were elongated. In young dogs superficial cells of epithelium had a lot of vacuoles. The stromal collagen formed different clearly seen layers in young and adult dogs' cornea. The corneal structure of the pigs' cornea is similar to that in dogs. Two types of cells, dark and light, were seen in the corneal epithelium of the piglets. Corneal stroma consisted of the collagen layers and fibroblasts. Descemet's membrane was seen as thick homogenous layer in the piglet cornea. The endothelial cells of piglets' cornea had more square form than adult pigs' cells.

Keywords: cornea structure, light and electron microscopy, dog, pig.

RAGENOS SANDAROS YPATUMAI: ŠVIESINĖ IR ELEKTRONINĖ MIKROSKOPIJA

Santrauka. Tyrimo tikslas buvo išanalizuoti ir palyginti jaunų ir suaugusių šunų bei kiaulių ragenos struktūrą naudojant šviesinę ir elektroninę mikroskopiją. Tirti jauni (10–15 dienų) ir suaugę (vyresni nei 8 mėn.) šunys bei jaunos (2 dienų) ir suaugę (8–9 mėn.) kiaulės. Ištirta 4 jaunų šunų akys ir po 12 suaugusių šunų, jaunų ir suaugusių kiaulių akių. Ragenos storis išmatuotas ultragarsiniu pachimetru. Ragena ištirta šviesine (abiejų grupių kiaulių ir suaugusių šunų) ir elektronine mikroskopija.

Ultragarsinis tyrimas parodė, kad kiaulių ragena buvo žymiai storesnė nei atitinkamo amžiaus šunų ragena. Suaugusių individų ragena buvo plonesnė nei jaunų.

Šviesinė mikroskopija parodė, kad suaugusių kiaulių ir šunų ragenoje gerai matoma Descemeto membrana paršelių ragenoje nesuformuoja aiškiai išskiriamo sluoksnio.

Jaunų ir suaugusių šunų rageną ištyrus elektroniniu mikroskopu matyti, kad ragenos epitelis sudarytas iš kelių ląstelių sluoksnių. Epitelio pamatinio sluoksnio ląstelių membrana gerai matoma ir jaunų, ir suaugusių šunų ragenoje. Paviršinio epitelio sluoksnio ląstelės ištęstos formos, jaunų šunų ląstelėse matomos vakuolės. Ragenos stromos kolageninės skaidulos suformuoja aiškiai matomus sluoksnius jaunų ir suaugusių šunų ragenoje. Kiaulių ragenos struktūra panaši į šunų. Ragenos epitelyje matomos dviejų tipų – šviesios ir tamsios – ląstelės. Ragenos stroma sudaryta iš kolageninių skaidulų ir fibroblastų. Paršelių ragenoje kaip storas homogeninis sluoksnis matoma Descemeto membrana. Paršelių ragenos endotelio ląstelės yra taisyklingesnės formos nei suaugusių kiaulių.

Raktažodžiai: ragenos struktūra, šviesinė ir elektroninė mikroskopija, šuo, kiaulė.

Introduction. The transparent nature of the cornea and its importance in the visual pathway as the major refracting lens of the eye have intrigued workers in many different disciplines and their studies have added immeasurably to the understanding of the cornea in health and disease (Beuerman, Pedroza, 1996). Evaluation of canine cornea using electron microscopy can be done in some virologic diseases (Rapp, Kolbl, 1995). Age related morphological changes of pigs' cornea and distribution of age-related localization of immunoreactive substance P in corneal nerve plexus of the dog were investigated (Babrauskienė, 1997; Lasys, Sienkiewicz et al., 2000).

The cornea is composed of five layers: anterior epithelium, subepithelial basement membrane, substantia propria or stroma, posterior limiting lamina (Descemet's membrane) and posterior epithelium (corneal endothelium). (Dellmann, Eurell, 1998). The number of cell layers in the epithelium varies with the species, as well as the location on the cornea (Gelatt, 1981; Harrison, Joos, Ambrosio, 2003). Morphometric characteristics such as thickness, the number of cellular layers and the number of the epithelial cells in different mammals' groups were examined. The study revealed the morphometric characteristics of the epithelium of primates and carnivores to have more similarities than those of herbivores (Merindano, Canals, Potau et al., 1998).

Corneal stroma consists of collagen fibrils (primarily type I collagen) embedded in mucopolysaccharide ground substantial well defined composition. Corneal transparency is the result of a small size of constituent collagen fibrils, smaller than the waves of visible light (400-700 nm) that are ordered arrangement in corneal lamellae (Clark, 2001). The lamellae of the posterior stroma are more regular in arrangement than those of the anterior one-third of the stroma (Freud, McCally, Farrell et al., 1995). The most anterior stroma has a thin, cell-free zone that corresponds in location to the anterior limiting membrane in primates. Fibroblasts contain extensive branching processes that may contact other fibroblasts (Meek, Leonard, 1993)

The mean radius of collagen fibrils, intermolecular spacing of human cornea was found to be age dependent. The age-related growth of fibril diameter was mostly a result of an increased number of collagen molecules and, in addition, to some expansion of the intermolecular spacing probably resulting from glycation-induced crosslinking (Daxer, Misof, Grabner et al., 1998; Malik, Moss, Ahmed et al., 1992)

In Descemet's membrane, fibrous long spacing (FLS) fiber-like structures, arranged in parallel to the endothelium were observed. SEM showed that Descemet's membrane has piled laminar structure. Descemet's membrane is closely associated with the collagen layer of the substantia propria. Collagen fibrils invading from the substantia propria into Descemet's membrane were observed (Hayashi, Osawa, Tohyama, 2002). The posterior limiting membrane (Descemet's membrane) is actually an exaggerated basal lamina of the posterior epithelium. It is produced by the posterior epithelium throughout life, thus forming a thicker membrane as animal ages (Gelatt, 1981).

The corneal endothelium is composed of a single layer of squamous cells and forms the endothelial layer of the anterior chamber of the eye. Some controversy exists over the regenerative ability of the posterior epithelium and indeed it may vary with species. The corneal epithelium has a remarkable capacity for regeneration and heals in approximately 7 days. In contrast, the endothelium in mature cornea has very low mitotic activity and responds to trauma primarily by cellular hypertrophy and migration (Arndt, Reese, Kostlin, 2001). The study of endothelial cells repairing of canine cornea showed reendothelialization occurred by both mitotic division and enlargement and migration of remaining undamaged cells. The corneal endothelium of the young adult dogs had a marked regenerative potential (Befanis, Peiffer, Brown, 1981). Dog corneal endothelium responds to surgical trauma in a manner similar to man and this can represent a good model study of corneal endothelial disease in man (Gwin, Warren at al., 1983).

The aim of this study was to examine and compare the cornea structure of young and adult dogs, and pigs using light and electron microscopy.

Materials and methods. Young (10-15-day-old) and adult (over 8 month) dogs, and young (2-day-old) and adult (8-9 month) pigs were used in this study. The principles of laboratory animal care (NIH publication No. 86-23, revised in 1985) and the specific national laws on protection of animals were followed. We used 4 eyes of young dogs, 12 eyes of adult dogs, 12 eyes of piglets and 12 eyes of adult pigs. Corneal thickness of the dogs were measured in vivo using ultrasonic pachymeter. Later cornea was taken for light (only from adult dogs) and transmission electron microscopy. Cornea of young and adult pigs was measured using ultrasonic pachymeter

after eyeball enucleation, and later material was taken for light and electron microscopy. Electron microscopy of the pigs corneas was done in KMU department of Anatomy whereas dogs corneas were examined in the department of histology and embryology of University of Warmia and Mozury in Olsztyn.

Technique of the light microscopy: 5x5 mm parts of the corneas were fixed in Buen's solution for 24 hours, dehydrated in series of ethanol solutions (50, 70, 80, 100%), released in the xylene and embed in paraffin. Sections were stained in hematoxylin-eosin. Slides were examined using light microscope ("Leitz").

Technique of the electron microscopy: central part of the dogs' cornea was fixed in 2.5 % solution of glutaraldehyde in phosphate buffer, pH 7.4, for 2 hours, at 4^{0} C temperature. Postfixation was done in 4% osmium tetroxide in phosphate buffer pH 7.4 for 2 hours followed by dehydration in a series of graded ethanol solutions (30, 50, 70, 80, 90, 95, 100%) and embed in Epon. Semithin sections were stained with methylen blue. Ultrathin sections were stained with lead citrate and uranyl citrate. Later sections were examined using transmission electron microscopy (Tesla).

Corneas of the pigs were fixed in 2% solution of glutaraldehyde in phosphate buffer, pH 7.3 for 12 hours. Postfixation was done in 1% osmium tetroxide in cacodylate buffer for 1 hour at 4° C temperature. Dehydration was done in the ethanol solutions (50, 80, 90, 100%) and acetone (100%) and embed in Epon and Araldit. Semithin sections were stained with methylen blue. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined using electron microscopy (Tesla and Zeiss).

Results. Ultrasonic measurements of cornea thickness showed that corneas of young and adult pigs were significantly thicker than corneas of young and adult dogs (P<0.05). Cornea of adult individuals was thinner than that young ones (Table 1).

Light microscopy showed all cornea layers clearly seen in the slides of the adult pigs' cornea. Endothelium of the adult dogs' cornea has not been seen in the light microscopy slides. Corneal epithelium contained several layers of the cells. The Descemet's membrane was seen in the adult pigs and dogs corneas, but in piglets cornea the definite layer did not mould (Fig. 1-2).

Electron microscopy of the dogs' cornea revealed that basal cells in adult dogs cornea were square with big nucleus in the center of the cell (Fig. 3). Cells of the young dogs cornea epithelium were more oval with big nucleus (Fig. 4). The basement membrane of the corneal epithelium cells was clearly seen in both, young and adult dogs cornea. Superficial cells of the cornea epithelium were elongated. In the young dogs superficial cells of epithelium have had a lot vacuoles. The peeling superficial cell as homogenous membrane was seen in the cornea electron pictures of young dogs (Fig. 4). The stromal collagen formed different clearly seen layers in young and adult dogs' cornea. Elongated fibroblasts, seen between collagen layers, contained big nucleus and endoplasmic reticulum (Fig. 5-6) The corneal structure of the pigs' cornea is similar to that in dogs. Two types of cells, dark and light were seen in the corneal epithelium of the piglets. Corneal stroma consisted of collagen layers and

fibroblasts. Descemet's membrane was seen as a thick homogenous layer in the piglet cornea (Fig. 7-8). The endothelial cells of piglets' cornea had more square form than adult pigs cells. The nuclei of endothelial cells were more regular in piglets than nuclei in the epithelium of the adult pigs. The endoplasmic reticulum, mitochon-dria and pynocytotic vesicles were seen in the endothelial cells of adult pigs (Fig. 9-10).

1	Table.	Thickness	of	the	cornea,	μm
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No.	10-15-days-old dogs	< 8 month dogs	2-days-old pigs	8-9 month pigs
	(n=4)	(n=12)	(n=12)	(n=12)
1.	629	416	1300	1100
2.	623	607	1300	1100
3.	657	525	1200	1100
4.	677	607	1100	1100
5.		606	1400	1100
6.		620	1100	1100
7.		540	1100	1100
8.		536	1200	1200
9.		574	1200	1100
10.		524	1300	1100
11.		719	1000	1100
12.		709	900	1100
Av.	646.5	581.92	1175	1108.3
SD	25.159	83.274	142.22	28.87

Av. - average; SD - standard deviation

Discussion. The aim of this study was to examine and compare the cornea structure of young and adult dogs, and pigs using light and electron microscopy.

The cornea of the dogs and pigs was found to contain the same layers: corneal epithelium, stroma, Descemet's membrane and corneal endothelium. The subepithelial basement membrane was not seen as separate layer in the corneas of these animals. Edothelium of the adult dogs' cornea was not seen in light microscopy slides. The Descemet's membrane was seen in the adult pigs and dogs' cornea, but in piglets' cornea the definite layer in the light microscopy slides did not mould.

Using SEM, light and dark cell types can be identified in the cornea epithelium. The light cells contain more numerous projections act to scatter electrons and produce the lighter appearance of the cell. The lighter cells are thought to be a younger cell type (Gelatt, 1981). These two types of cells we could see in the corneal epithelium of piglets but there were no cell separation in the cornea of young and adult dogs, and adult pigs. The Bowman's layer of the cornea was hardly indicated in the dogs and pigs' cornea electron microscopy slides, but the basement membrane of the corneal epithelium cells was clearly seen in both young and adult dogs cornea. Superficial cells of the cornea epithelium were elongated. In the young dogs superficial cells of epithelium had a lot of vacuoles. The peeling superficial cell as homogenous membrane was seen in the cornea electron pictures of young dogs.

The evaluation of the toxicity of ethanol on the rabbit corneal epithelium using electron microscopy showed widespread partial or total damage of microvilli, focal breaks of intercellular junction and cellular edema. Increasing exposure time to ethanol over 1 minute resulted in significant damage to superficial corneal epithelium and prolonged its normal recovery time (Kim, Sah, Lim et al., 2002).

The substantia propria forms the major bulk of the cornea and is composed of collagen fibers arranged in bundles (lamellae), fibroblasts and ground substances. The lamellae are parallel bundles of collagen fibrils. The collagen fibrils within lamellae are all parallel, but between lamellae they vary greatly in directions (Gelatt, 1981). These bunds of lamellae of collagen fibrils were seen as the layer of corneal stroma, and in the electron microscopy cut they vary in the direction of dogs and pigs'cornea. In the cross section of lamellae the regular diameter could be indicated. Elongated fibroblasts, seen between collagen layers, have contained big nucleus and endoplasmic reticulum.

Descemet's membrane was poorly indicated using light microscopy, but clearly seen in the electron microscopy slides of pigs' cornea.

The regenerative capabilities of cat endothelium are limited and that maintenance of corneal dehydration involves cell enlargement and migration, rather than cell division. The corneal endothelium is essential for corneal transparency by virtue of a physiologic pump mechanism that moves stromal fluid into the aqueous humor, maintaining a state of relative stromal dehydration and normal corneal thickness. Endothelial cells of central part of cat cornea formed a mosaic-like pattern of hexagonal cells 15 to 20 µm in diameter; the average number of cells per millimeter was 2.668±211SD (Peiffer, De Vanzo, Cohen, 1981). The morphology of corneal endothelium is not only affected by trauma but also by ageing. In people over 50 years of age, the endothelial cells are not consistently hexagonal in shape, but rather pleomorphic, so both small and large cells occur. The number of endothelial cells decrease with age, and larger cells appear to result from cell loss that is compensated by cellular hypertrophy (Arndt, Reese, Kostlin, 2001). Canine corneal endothelial cells appear quite similar to those of other species. The hexagonally shaped canine endothelial cells tend to

enlarge with age, with population in young animals averaging around 2500 cells/mm² and the number of cells in older dogs being frequently below 2100 cells/mm². A significant increase in corneal thick-ness was observed with age (Gwin, Lerner et al., 1982). The endothelial cells of piglets' cornea had more square form than adult pigs' cells. The decreased number of cells in endothelium could condition a loss of the square shape of the cell in adult pigs cornea. The endoplasmic reticulum, mitochondria and pynocytotic vesicles were seen in the endothelial cells of adult pigs. These structures indicate involvement of endothelial cells in secretion and active transport and thus it's not surprising that their primary function is to nourish and hydrate the cornea. With disturbance in endothelial function, water diffuses into the stroma and disrupts the



Figure 1. Cornea of the 2-days-old piglets. Light microscopy, HE (10x40)

1 – epithelium, 2 – stroma or substantia propria, 3 – endothelium, 4 – basal cells layer of epithelium.

parallel arrangement of collagen fibrils resulting in cornea opacity.

The decreased cell density in corneal epithelium plays a significant role in transplantation. The higher the endothelial cell density is at the time of transplantation, the greater the chance that the cornea will remain transparent. Whereas the endothelium is responsible for fluid transport the corneal thickness provides information about the hydration status of the cornea and changes of endothelial cells (Arndt, Reese, Kostlin, 2001).

Ultrasonic measurements of corneal thickness revealed cornea of young individuals to be thicker than that of adult ones. The lens of young animals is near the posterior surface of the cornea and the measurement of cornea can involve lens thickness.





1 – epithelium, 2 – stroma or substantia propria, 3 – endothelium, 4 – basal cells layer of enpithelium, 5 – Descemet's membrane.



Figure 3. Corneal epithelium of the adult dogs (x8000) 1 - detaching superficial cell; 2 - cell of the superficiallayer, 3 - cell of the basal layer; 4 - nucleus, 5 basement membrane of the corneal epithelium



Figure 4. Corneal epithelium of the young dog (x8000) 1 – detaching superficial cell, 2 – intercellular spaces



Figure 5. Corneal stroma of the adult dogs (x 8000). 1 – fibroblast, 2 – nucleus, 3 – collagen layers



Figure 6. Corneal stroma of the young dogs (x6000) 1 – nucleus; 2 – fibroblast, 3 – endoplasmic reticulum, 4 – collagen layers



Figure 7. Corneal epithelium of the piglets (x8000) 1 – extracellular formations, 2 – nucleus, 3 – light cell; 4 – dark cells



Figure 8. **Corneal stroma of the piglets (x8000)** 1 – *fibroblast; 2 – collagen layers*



Figure 9. Corneal endothelium of the adult pigs (x8000) 1 – endothelial cell, 2 – nucleus, 3 – pynocitotic vesicles, 4 – Descemet's membrane



Figure 10. Corneal endothelium of the piglets (x8000) 1 - endothelial cell, 2 - nucleus, 3 - Descemet's membrane

References

1. Arndt C., Reese S., Kostlin R. Preservation of canine and feline corneoscleral tissue in Optisol®GS. Veterinary ophthalmology. 2001. Vol. 4, No. 3. P. 175-182.

2. Babrauskienė V. Kiaulių akių ragenos struktūros amžinių pokyčių analizė. Veterinarija ir zootechnika. Kaunas, 1997. T. 3 (25). P. 13-14.

3. Befanis P.J., Peiffer R. L., Brown D. Endothelial repair of the canine cornea. Am J Vet Res. 1981. Vol. 42. P. 590-595.

4. Beuerman R. W., Pedroza L. Ultrastructure of the human cornea. Microscopy research and technique. 1996. Vol. 33. P. 320-335.

5. Clark J. I. Fourier and power law analysis of structural complexity in cornea and lens. Micron. 2001. Vol. 32. P. 239-249.

6. Daxer A., Misof K., Grabner B et al. Collagen fibrils in the human corneal stroma: structure and aging. Invest Ophthalmol Vis Sci. 1998. Vol. 39. P. 644-648.

7. Dellmann H. D., Eurell J. A. Textbook of veterinary histology. Williams and Wilkins. 1998. P. 333-344.

8. Freud D. E., McCally R. L., Farrell R. A. et al. Ultrastructure in anterior and posterior stroma of perfused human and rabbit corneas. Relation to transparency. Invest Ophthalmol Vis Sci. 1995. Vol. 36. P. 1508-1523

9. Gelatt K. N. Textbook of veterinary ophthalmology. Philadelphia: Lea & Febiger, 1981. P. 23-35.

10. Gwin R. M., Lerner I., Warren J. K. et al. Decrease in canine corneal endothelial cell density and increase in corneal thickness as functions of age. Invest Ophthalmol Vis Sci. 1982. Vol. 22, No. 2. P. 267-271.

11. Gwin R. M., Warren J. K., Samuelson D. A. et al. Effects of phacoemulsification and extracapsular removal on corneal thickness and endothelial cell density in the dog. Invest Ophthalmol Vis Sci. 1983. Vol. 24. P. 227-236.

12. Harrison D. A., Joos C., Ambrosio R. Morphology of corneal basal epithelial cells by in vivo slit-scanning confocal microscopy. Cornea. 2003. Vol. 22. P. 246-248.

13. Hayashi S., Osawa T., Tohyama K. Comparative observations on corneas, with special reference to bowman's layer and descemet's membrane in mammals and amphibians. J. Morphol. 2002. Vol. 254. P. 247-258.

14. Kim S.Y., Sah W. J. Lim Y. W. et al. Twenty percent alcohol toxicity on rabbit corneal epithelial cells. Cornea. 2002. Vol. 21. P. 388-392.

15. Lasys V., Sienkiewicz W., Stropus R. et al. The distribution and age-related localisation of immunoreactive substance P in corneal nerve plexus of the dog. Polish Journal of Veterinary sciences. 2000. Vol. 3, No. 2. P. 141-146.

16. Malik N. S., Moss S. J., Ahmed N. et al. Ageing of the human corneal stroma: structural and biochemical chamges. Biochim Biophys Acta. 1992. Vol. 1138. P. 222-228.

17. Meek K. M., Leonard D. W. Ultrastructure of the corneal stroma: a comparative study. Biophys J. 1993. Vol. 64. P. 273-280.

18. Merindano M. D., Canals M., Potau J. M. et al. Morphometrical features of the corneal epithelium in mammals. Anat Histol Embryol. 1998. Vol. 27, No. 2. P. 105-110.

19. Peiffer R. L., De Vanzo R. J. Cohen K. L. Specular microscopic observations of clinically normal feline corneal endothelium. Am J Vet Res. 1981. Vol. 42, No. 5. P. 854-855.

20. Rapp E., Kolbl S. Ultrastructural study of unidentified inclusions in the cornea and iridocorneal angle of dogs with pannus. Am J Vet Res. 1995. Vol. 56, No. 6. P. 779-785.

21. Slatter D. Fundamentals of veterinary ophthalmology. Philadelphia: W. B. Saunders Company, 1990. P. 257-269.