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ABSTRACT

of the dissertation for the degree of Doctor of Philosophy

**ULTRASTRUCTURAL AND IMMUNOHISTOCHEMICAL
CHARACTERIZATION OF THE STRUCTURES OF THE
BRAIN MENINGES ACTING AS A BIOLOGICAL BARRIER
AND THE CHANGES OCCURRING DURING
EXPERIMENTAL EDEMA**

Speciality: 2407.01 – “Cell biology, cytology, and
histology”

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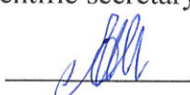
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GENERAL CHARACTERISTICS OF WORK

Relevance of the problem and the degree of development. The intrafascicular structural organization of the components of the peripheral nervous system, the morphofunctional characteristics of nerve fibers and their age-related features, as well as the branching patterns of major vessels and microvessels related to local blood circulation, have also been studied by national researchers.^{1,2}

One of the common features uniting almost all structures of both the central and peripheral nervous systems is that they are surrounded by meningeal elements that differ from one another in their structural organization. According to recent data, the meninges, in addition to their covering (protective) function, are directly involved in the circulation of cerebrospinal fluid the histogenesis of neural elements, and the maintenance of the stability of the interstitial environment essential for their normal functioning, both directly and through the biologically active substances they synthesize.³ Despite these observations, disagreements still persist regarding the interactions of the structures involved in the organization of the meninges, their ultrastructural characteristics, and their roles in performing specific functions. Some modern sources note that the meninges are a multilayered structure composed solely of fibroblasts, containing blood vessels, lymphatic vessels, and immune cells. At the same time, there is information indicating that the meningeal elements covering various parts of the central nervous system are primarily epithelial in structure and derive from the ectoderm (neural crest). Among the bacteria belonging to the Enterobacteriaceae family, the rod-shaped,

¹ Шадлинский, В.Б., Гусейнов, Б.М., Мустафаева, Н.А. (Shadlinskiy, V.B., Huseynov, B.M., Mustafayeva, N.A.) Варианты ветвления подглазничного нерва // Морфология, – 2016. т. 150, №4, – с. 55-58.

² Гасымов, Э.К. (Gasimov, E.K.) Пучки нервных волокон как транспортная единица в стволе периферических нервов // Азербайджанский медицинский журнал, – 1996. №9, – с. 14-18.

³ Stolp, H.B., Liddelow, S.A., Saunders, N.R. Editorial: ontogeny and phylogeny of brain barrier mechanisms // *Frontiers in Neuroscience*, – 2016. 10(41), – p. 1-3.

facultatively anaerobic, Gram-negative intestinal rods called *Escherichia coli*, which inhabit the distal parts of the intestines of warm-blooded animals, serve as a reference standard under both clinical and experimental conditions. *E. coli* has a rich history as the "rock star" of biology and holds invaluable significance in achieving major innovations across many fields of biology and medical sciences. In nature, *E. coli* harmlessly inhabits the intestines of humans and animals, but when this balance is disrupted for various reasons, it can cause approximately 4,000 infections and lead to the development of 900 cases of hemolytic-uremic syndrome.⁴ Although the perineural barrier has been studied in detail, information on the structures that serve as biological barriers at the level of the meninges is still insufficient. Analysis of the available literature indicates that, because the primary focus during the creation of experimental edema models of various origins has been on changes occurring in the vascular system, the structural alterations in the cerebral meninges caused by *E. coli* endotoxin have not been sufficiently studied.⁵ The parenchyma of the nervous system is functionally separated from immunocompetent cells that may enter from the blood by a system of multilayered cellular and non-cellular barriers, which isolate neurons, glial elements, and nerve fibers. Within these boundaries, the meninges of the brain, spinal cord, and peripheral nerves, which act as dynamic observers and play a crucial role in maintaining organ homeostasis, have not received sufficient attention regarding the resident macrophages.

Aim and objectives of the study. To clarify the histotopography, ultrastructural and immunohistochemical characteristics of the parts of the meninges that serve as biological barriers and their surrounding

⁴ Foster-Nyarko, E., Pallen, M.J. The microbial ecology of *Escherichia coli* in the vertebrate gut // FEMS Microbiology Reviews, – 2022. 46(3), – p. 1-22.

⁵ Barichello, T. Neonatal *Escherichia coli* K1 meningitis causes learning and memory impairments in adulthood / Tatiana Barichello, Valdemira Dagostim, Jaqueline Generoso [et al.] // Journal of Neuroimmunology, – 2014. 272(1-2), – p. 35-41.

structures across various derivatives of the central and peripheral nervous systems, as well as their involvement in the distribution of micromolecular tracers and the changes that occur during acute endotoxemia.

Objectives of the study:

1. To determine the histotopography and ultrastructural characteristics of the structural elements that serve as biological barriers in the meninges of the brain and their surrounding structures;
2. To study the histotopography and ultrastructural characteristics of the structural elements that serve as biological barriers in the meninges of the spinal cord and their surrounding structures;
3. To clarify the ultrastructural characteristics of the structural elements that serve as biological barriers in the meninges of peripheral nerves;
4. To investigate the histotopography and ultrastructural characteristics of the blood vessels located in the meninges of the brain, spinal cord, and sciatic nerve;
5. To precisely determine, at the ultrastructural level, the topographic positions of the meningeal elements that serve as barriers using micromolecular tracers;
6. The immunohistochemical characterization of the meningeal elements that serve as barriers;
7. To identify the changes occurring in the meningeal elements covering different parts of the nervous system and in the vessels involved in their nourishment in an acute experimental endotoxemia model;

Methods of the study. In the scientific research work, light, electron microscopic, immunohistochemical, variational, and non-parametric statistical methods were used to study the meningeal elements of the nervous system in mammals (white rat) and birds (domestic duck and goose).

Scientific novelty of the study:

1. The corresponding and distinct ultrastructural features of the brain and spinal meninges in birds and mammals have been clarified.

2. Species-specific characteristics of the histotopography and ultrastructural organization of the structures serving as biological barriers have been identified.

3. The relationship between the immunohistochemical parameters of the meningeal elements serving as biological barriers and their ultrastructural characteristics has been revealed.

4. The anisotropic distribution of micromolecular tracers at the levels of the spinal cord and sciatic nerve meninges has been clarified ultrastructurally.

5. The relationships between pathological changes during acute endotoxemia and the histogenesis, topographic position, and ultrastructural characteristics of various cell types have been determined.

Main statements submitted for defense:

- Similar and distinct features of the histotopography, immunohistochemical, and ultrastructural organization of the cellular and non-cellular elements serving as biological barriers;

- The presence of lymphatic vessels incorporated into the microcirculation system in various parts of the brain and spinal meninges and peripheral nerve trunks;

- The existence of structures that do not serve as biological barriers at the level of meningeal elements covering different parts of the central and peripheral nervous systems;

- The role of edema fluid in the destructive changes occurring in barrier structures during acute endotoxemia;

- Ultrastructural evidence of the transport of tannic acid molecules, as micromolecular tracers, into the inner layers of barrier cells in the spinal cord and sciatic nerve;

- The effect of the anisotropic distribution of micromolecular tracers at the meningeal level on cerebrospinal fluid and endoneural fluid circulation.

Theoretical and practical significance of the study.

- The factual material obtained through the application of complex methods used in the study allows for the identification of

structures that serve as biological barriers in other parts of the organism.

- The electron microscopic and immunohistochemical data obtained on meningeal elements with different topographic positions and structures can facilitate the detection of malignant tumors involving these structures, their metastases, and the refinement of differential diagnoses.

- It can be used in selecting treatment principles aimed at restoring the functions of cellular elements most affected during acute endotoxemia.

Application of the results. The results obtained in the study have been applied in practice at the departments of Human Anatomy and Medical Terminology, Pathological Anatomy, Neurology, and Cytology, Embryology and Histology of the Azerbaijan Medical University.

Approval and application. The materials of the dissertation were presented and discussed at meetings and seminars of the Department of Cytology, Embryology and Histology of the Azerbaijan Medical University (2012–2024); at the joint congress of the Peripheral Nerve Society (PNS, USA) and the Inflammatory Neuropathy Consortium (Rotterdam, the Netherlands, 2012); at the XXI International Congress of Neurology (Vienna, 2013); at the IX International Congress of the Federation of European Neurological Societies (FENS) (Milan, 2014); at the Baku International Humanitarian Forum (Baku, 2014); at the Scientific and Practical Conference of Azerbaijan Medical University “Actual Problems of Medicine” dedicated to the 100th anniversary of the Azerbaijan Democratic Republic (Baku, 2018); at the International Scientific and Practical Conference dedicated to the 100th anniversary of the establishment of the Faculty of Medicine (Baku, 2019); at the Scientific Conference dedicated to the 90th anniversary of the birth of Corresponding Member of ANAS, Honored Scientist, Professor Demir Vahid oğlu Hacıyev (Baku, 2019); at the International Scientific and Practical Congress of Azerbaijan Medical University “Actual Problems of Medicine” dedicated to the 100th anniversary of the

National Leader Heydar Aliyev (Baku, 2023); at the XXVI Congress on Electron Microscopy (Eskisehir, Turkey, 2023); and at the International Scientific and Practical Congress “Actual Problems of Medicine” (Baku, 2024), at the joint meeting of the staff of the Departments of Cytology, Embryology and Histology, Human Anatomy and Medical Terminology, Medical Biology and Genetics, Pathology, and the Scientific Research Center of Azerbaijan Medical University (Baku, 15.05.2025, protocol №1), at the scientific seminar of the Dissertation Council FD 2.08 under Azerbaijan Medical University of the Higher Attestation Commission under the President of the Republic of Azerbaijan (Baku, 26.05.2026, Protocol №1).

Name of the organization where the dissertation was conducted. The research was conducted at the Department of Cytology, Embryology, and Histology of Azerbaijan Medical University.

Structure and volume of the dissertation. The dissertation consists of 234 pages, with a total text part of 239740 characters, consisting of an introduction (12785 characters), 6 chapters (Chapter I: “Literature review” - 43024; Chapter II: “Materials and methods of research” - 8735; Chapter III: “Histotopography and electron microscopic structural features of membrane elements covering various parts of the nervous system” - 96815; Chapter IV: “Comparative characteristics of immunohistochemical and electron microscopic properties of the cerebral and spinal cord membranes” - 9479; Chapter V: “Electron microscopic study of the distribution of tannic acid as a micromolecular tracer at the level of membranes covering the spinal cord and peripheral nerves” - 5882; Chapter VI: “Light and electron microscopic study of the effect of *E. coli* endotoxin on structural elements of the cerebral membranes” - 19799 characters), conclusion (38369 characters), results (3896 characters), practical recommendations (956 characters) and a list of literature.

The work includes 74 figures, 2 tables, 257 literary sources, including 16 in Azerbaijani, 18 in Russian, and 223 in English.

MATERIALS AND METHODS OF RESEARCH

The study was conducted on tissue samples obtained from the brains, spinal cords, and sciatic nerves of 45 white rats, 15 domestic ducks, and 6 domestic geese (a total of 66 animals). Electron microscopic investigations were carried out on tissue samples taken from the corresponding regions of 15 white rats, 6 domestic ducks, and 6 domestic geese. Based on the methods adopted for electron microscopy, Araldite-Epon blocks were prepared from the samples.⁶ Semithin sections (1–2 μm) obtained from the blocks using a EM UC7 (Leica) ultramicrotome were stained by a single-step staining method, examined under a Primo Star (Zeiss) microscope, and images of the required areas were captured with an EOS D650 (Canon) digital camera. Ultrathin sections (50–70 nm) obtained from the same blocks were stained with a 2% uranyl acetate solution, and 0.2% lead citrate. The ultrathin sections were examined under a JEM-1400 transmission electron microscope at 80–120 kV, and electronograms were captured. Morphometric analysis of the images was performed on the electronograms saved in TIF format using the computer software The TEM Imaging Platform. A 1% tannic acid solution prepared in 0.5 M cacodylate buffer (pH 7.4) was used as a micromolecular tracer. In domestic ducks, the solution was administered into the epidural space within the spinal canal, while in white rats, it was applied around the sciatic nerve. After fixation of the respective structures with 2.5% glutaraldehyde solution, post-fixation was carried out in 1% osmium acid solution. Blocks were prepared from tissue samples taken 1 and 2 hours after the administration of the 1% tannic acid solution in 0.5 M cacodylate buffer (pH 7.4) to the perispinal and perisciatic areas, including white matter of the spinal cord and fragments of the sciatic nerve, and these blocks were then examined.

⁶ Kuo, J. Electron microscopy: methods and protocols / J. Kuo. – Totowa: Humana Press, – 2014, – 799 p.

Immunohistochemical studies were carried out on the meningeal elements covering the brains and spinal cords of six domestic ducks. Following fixation of the samples in a 10% neutral formalin solution and standard paraffin embedding, histological sections were prepared. After antigen retrieval and quenching of endogenous peroxidase activity, pancytokeratin, EMA, vimentin, and glial fibrillary acidic protein (GFAP) were applied to the sections and incubated with primary antibodies. For visualization, the sections were incubated sequentially in biotin (Sensi Tek Anti-Polyvalent Biotinylated Antibody) for 30 minutes, avidin-streptavidin (Sensi Tek HRP) for 30 minutes, and DAB (Diaminobenzidine Chromogen) solution for 10 minutes. This step was omitted in the control group. Following nuclear counterstaining with Mayer's hematoxylin, the sections were mounted with balsam and coverslipped. The obtained results were evaluated as positive (+) or negative (-). To create an acute endotoxemia model, 20 adult white rats weighing 200–260 grams were divided into two groups. In the first group, 10 rats were administered purified LPS (Serotype 0111:B4; *In vivo* Gen, San Diego, USA, No. 78H4086) at a dose of 1 mg/kg, dissolved in 0.5 ml of physiological saline, via the tail vein to induce experimental endotoxemia. In the second, control group, 10 rats were administered only 0.5 ml of physiological saline via the tail vein. Two hours after the start of the experiment, the brains, meninges, spinal, and sciatic nerves, along with surrounding structures, were fixed under general anesthesia using surgical instruments in both the control and experimental groups, and Araldite-Epon blocks were subsequently prepared. The morphometric parameters were analyzed using variation-statistical methods. After compiling variation series based on the obtained morphometric data, the maximum and minimum values, the difference between them (amplitude - R), and the arithmetic mean (X) were determined using the express-calculation tables of statistical indicators published by B.G. Kaplan (1970).⁷ Non-parametric statistical analyses of the

⁷ Каплан, Б.Г. Экспресс-расчет основных математико-статистических показателей / Б. Каплан. – Баку: Маариф, – 1970, – 446 с.

morphometric parameters of the elastic and collagen fibers participating in the organization of the spinal meninges and denticulate ligaments (Friedman test and Kendall's coefficient of concordance) were performed using GraphPad Prism 10.4.0 software. The obtained data were analyzed using variation statistics and box and whisker plots.

RESEARCH RESULTS AND DISCUSSION

The main aim of the study is to investigate the cells within the meningeal elements covering the brain, spinal cord, and sciatic nerve that serve as biological barriers along with the surrounding structures using light microscopy, electron microscopy, immunohistochemical methods, and tracer techniques and to obtain relevant information on the changes that occur during experimental endotoxemia. Both the brain and spinal cord are externally covered by three meninges: dura mater, arachnoid mater, and pia mater; the peripheral nervous system, in turn, is surrounded by epineurium, perineurium, and endoneurium. In all the research objects, the DM of the brain and spinal cord was found to contain fibrocytes and mononuclear cells with various functional activities, as well as cells distinguished ultrastructurally by a predominance of heterochromatin structures with peripheral localization, a high nucleus-to-cytoplasm ratio, and a sharply reduced number of organelles in the cytoplasm. Cells with the corresponding ultrastructural features were immunohistochemically found to express nestin, Sox2, doublecortin, and vimentin, and were identified as progenitor cells capable of differentiating into neural cells *in vitro* and *in vivo* after transplantation.⁸

It should be noted that in white rats, the DM of the brain draws attention due to a significantly lower number of fibrocytes and collagen fiber bundles, not only compared to birds, but even compared to the DM elements surrounding the rat's optic nerve.⁹

⁸ Bifari, F. Meninges harbor cells expressing neural precursor markers during development and adulthood / Francesco Bifari, Valeria Berton, Annachiara Pino [et al.] // *Frontiers in Cellular Neuroscience*, – 2015. 9(383), – p. 1-11.

⁹ Гасымов, Э.К., Оруджев, М.Т. Ультраструктурная организация паутинной оболочки зрительного нерва белой крысы // *Азербайджанский Медицинский Журнал*, – 2006. №2, – с. 99-102.

In the DM of the studied birds, the microvessels involved in microcirculation, originating from the arteries, are represented only by capillaries, characterized by fenestrations in the internal endothelial layer, while venular vessels are additionally characterized by open intercellular junctions. For the first time, all structures involved in lymph circulation (capillaries, postcapillaries, lymph valves defining their boundaries, and lymphatic vessels containing a single layer of smooth muscle cells in the wall) were identified in the DM of geese, and their ultrastructural characteristics were determined by electron microscopy. In addition, in the DM of domestic geese (which had not undergone any inflammatory or degenerative changes), postcapillary venules with high endothelium, involved in the recirculation of immunocompetent cells (mainly lymphocytes), were also observed. The results of the first scientific studies on the brain meninges using electron microscopy were devoted to clarifying two issues: 1) whether a subdural space involved in fluid circulation exists in the absence of any pathological changes; 2) what defines the boundary between the DM and the AM.

In white rats, one of the challenges encountered at the ultrastructural level is determining the topographic position of the basal lamina (indicated by arrowheads in Fig. 1A) between ABCL and ARCL. During the reconstruction of electronograms magnified 30,000 times, it was found that the described basal lamina is discontinuous rather than continuous. In areas where the basal lamina was not detected, the location of CFB, regardless of their size, should be considered as the boundary between the cellular barrier and the mesh layer. In this electronogram, of particular note in the ABCL, at the first two (adjacent) cells, in regions where tight junctions are located (indicated by wide arrows), adhesion points formed by the close apposition of plasmalemmas are observed, and the intercellular spaces measuring 20 nm between them are not detected. Sometimes, various widths (marked with an asterisk in Fig. 1B) are observed between adhesion points characteristic of tight junctions. It should be noted that recent published data have demonstrated the existence of the DBCL of

various mammals.¹⁰ In addition, in electron microscopic images of the AM of adult mice, all cells located above the LMC that are in contact with the basal lamina are assigned to the DBCL.¹¹

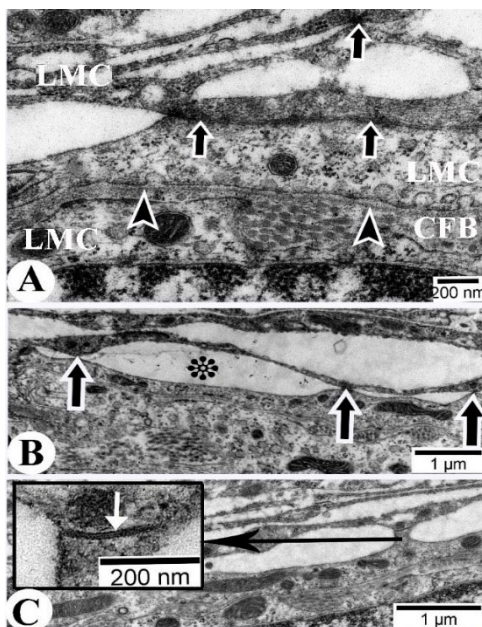


Figure 1. Electron microscopic features of structural elements attributed to the ABCL in white rats.

Based on the obtained data, it can be noted that the cells assigned to the DBCL, being in close contact with each other like the LMC, and connected through communicational junctions (indicated by an arrow in Fig. 1C), desmosomes, and, in certain regions, tight junctions, indicate that they are also part of the arachnoid barrier cell layer

¹⁰ Kinaci, A. Histologic comparison of the dura mater among species / Ahmet Kinaci, Wilhelmina Bergmann, Ronald Bleys [et al.] // Comparative Medicine, – 2020. 70(2), – p. 170-175.

¹¹ Pietilä, R. Molecular anatomy of adult mouse leptomeninges / Riikka Pietilä, Francesca Del Gaudio, Liqun He [et al.] // Neuron, – 2023. 111(23), – p. 3745-3764.

(ABCL). Starting from the first LMC layer of the ABCL, the pronounced compaction of cytoskeletal elements, condensation of chromatin structures involved in nuclear organization, the complete absence of nuclei in surface layer cells, and the presence of only occasional ribosomes and mitochondria in a state of destruction should be considered as a sign of keratinization with epithelial organization. In all research subjects, the presence of a subdural space capable of participating in the flow of biological fluids between the DM and AM elements was established histologically, immunohistochemically and by electron microscopy. As noted by some researchers, it can be fully agreed that, like epidural spaces, subdural spaces appear only during pathological processes.¹²

Another distinctive feature of the brain and spinal cord meninges in birds is the formation of a thick osmophilic line (indicated by arrowheads in Figs. 2A and 2B) at the ABCL–ARCL boundary, resulting from the close arrangement of the plasmalemmas of adjacent LMCs and basal lamina elements. In some regions of this thick osmophilic line, the cytoplasm of neighboring leptomeningeal cells are connected to each other through communicational junctions (shown with wide arrows in Fig. 2C) and desmosomes. The numerous finger-like processes of the leptomeningeal cells (marked as LMCs in Fig. 2B) within the barrier layer interconnect via adhesion points, forming a complex network of tight connections.

In the studied birds, denticulate ligaments were identified at the level of the spinal cord meninges and examined at both light and electron microscopic levels. At the ultrastructural level, it was established that, starting from the subarachnoid angle region, the denticulate ligaments are surrounded exclusively by the ARCL. Thus, there are generally no structures that would prevent (act as a barrier) the transport of CSF flowing between the ARCL trabeculae in the direction of the DM. In addition, at the level of the subarachnoid

¹² Ghannam, J.Y. Neuroanatomy, Cranial Meninges / J.Y. Ghannam, K.A. Al Kharazi, – Treasure Island, Stat Pearls, – 2024, – 48 p.

angles formed at the sites where the anterior roots of the spinal nerves abut the spinal ganglion, cerebrospinal fluid-conducting channels surrounded by extensions originating from the AM were identified. In conclusion, it should be noted that there are morphological bases for the penetration of CSF from the subarachnoid spaces into the venous and lymphatic vessels within the dura mater of the spinal cord through denticulate ligaments and the cerebrospinal fluid-conducting channels located in the subarachnoid angle.

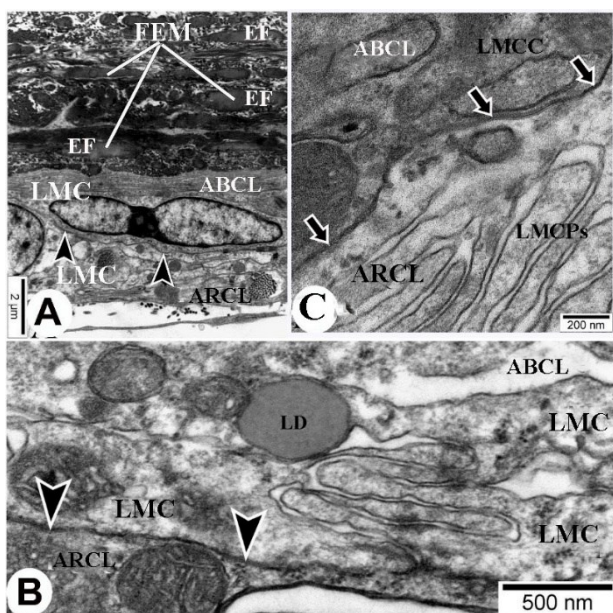


Figure 2. Electron microscopic features of cellular and fibrillar elements involved in the organization of the spinal cord in geese (A), ducks (B), and cranial meninges in geese (C).

In domestic ducks, differences were identified in the expression of type I, II, and III intermediate filament proteins in the brain, spinal cord, and the meningeal elements surrounding them. One of the issues raising questions among the obtained data is the absence of detectable expression of the vimentin protein (Clone V9) in the DM of the brain,

even under high magnification of the light microscope. The examination of electron micrographs shows that the perinuclear regions and most parts of the cytoplasmic processes of the intermediate filaments composed of vimentin protein in the cellular elements of the DM (mainly fibrocytes) vary in thickness between 100 and 300 nm. Thus, the fact that the perinuclear regions and protrusions of fibrocytes in DM are smaller than 400 nm makes it physically impossible to detect cytoskeletal elements under a light microscope. While the thickness of the fenestrated elastic membrane in column A of the statistical data (Figure 3, on the left) is greater only than the diameter of the terminal part of the denticulate ligament, the fact that the largest diameter of the elastic fibers corresponds to the origin (B) of the denticulate ligament, the medium diameter to the subarachnoid space (C), and the smallest diameter (D) to the level of the pia mater fully corresponds to the anatomical decrease in the diameter of the denticulate ligament as it branches. Thus, this provides full grounds to confirm that the elastic fibers participating in the formation of the denticulate ligaments originate not from the pia mater, but from the fenestrated elastic membrane of the dura mater.

It should be noted that in the chapter of the dissertation dedicated to materials and methods, the indicators for the Friedman test and Kendall's coefficient of concordance (Kendall's W), which pertain to non-parametric statistical analyses, were obtained through specific formulas using GraphPad Prism 10.4.0 software.

Calculations based on the studied materials show that the indicators for both the Friedman test and Kendall's coefficient of concordance reached their maximum values of 9 and 1, respectively.

To demonstrate the variability of the Friedman test indicators at the studied levels of elastic fibers, box-and-whisker plots were created (Figure 3, on the right). As seen from the figure: the non-central position of the median lines (especially across all three levels of the denticulate ligament - B, C, D) indicates that the data are asymmetrical, and the large size of the boxes (interquartile ranges) indicates a high degree of data variability. The proximity of the medians to the lower edge of the box in the initial and middle sections

along the course of the denticulate ligament indicates positive asymmetry, while in the lower section, it indicates negative asymmetry. In conclusion, it should be noted that despite the limited number of ducks ($n = 3$), the Friedman test showed that the overall difference in the diameters of the elastic ligaments among the four anatomical levels was statistically significant ($Q = 9.00$; $df = 3$; exact $p=0.0017$).

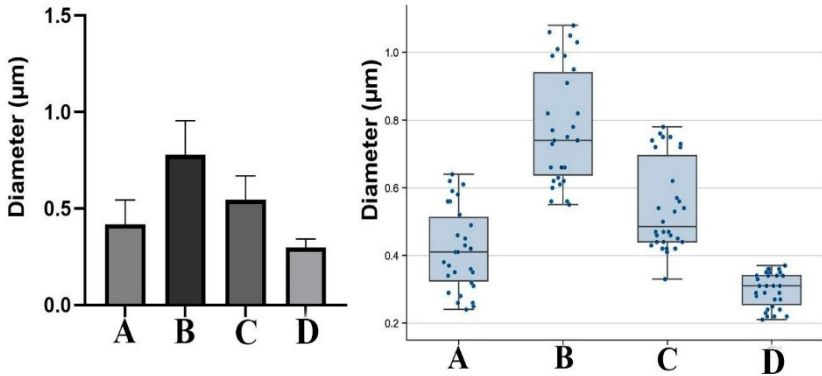


Figure 3. Statistical (left) and box and whisker (right) plots of the diameters of elastic fibers located in the fenestrated elastic membrane of the dura mater (A) and the initial (B), middle (C), and terminal (D) parts of the denticulate ligament of ducks.

It is noteworthy that in all three animals, the diameter of the elastic fibers demonstrated the exact same ranking sequence ($B > C > A > D$), which provides strong grounds to state that there is complete agreement among the rankings (Kendall's $W = 1.00$). The fact that both the Friedman test (indicating the difference between the studied groups of elastic fibers) and Kendall's coefficient (showing the agreement among the groups) have maximum values in the same structure (9 and 1, respectively) provides a basis for stating that the denticulate ligaments are of particular importance in maintaining the topographic and functional stability of structures located within the dura mater of the spinal cord under various conditions of the organism.

At the level of the brain AM, pan-cytokeratin expression was observed, unlike vimentin. Although staining with the primary antibody against pan-cytokeratin appears generally strongly positive under low magnification (Fig. 4A), under high magnification, areas with varying staining intensity as well as regions that are completely unstained (indicated by arrows in Fig. 4B) were observed .

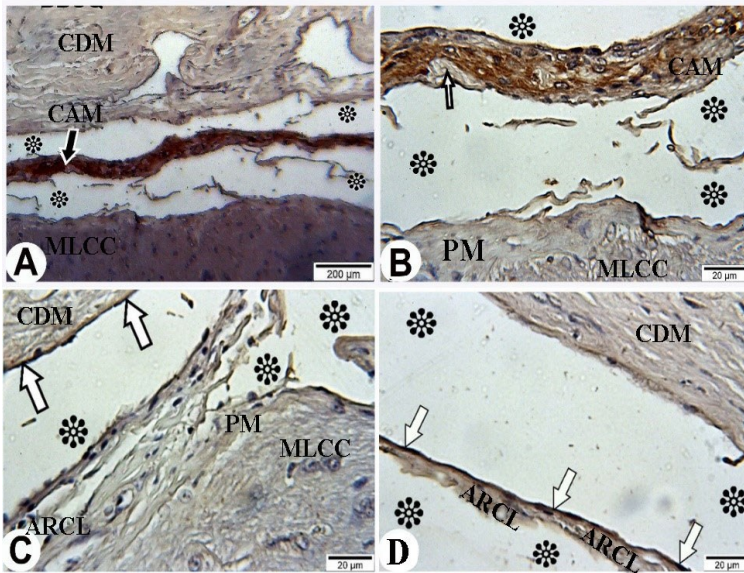


Figure 4. Microscopic images of immunohistochemical activity of intermediate filament proteins [Pancytokeratin (Clone AE1/AE3) and EMA (Clone E29)] in the meninges of domestic ducks. Image B is an enlarged fragment of image A, and image D is an enlarged fragment of image C.

Examination of the electron microscopic image of the described meninx shows that the unstained areas correspond to the locations of the collagen fiber bundles identified at the level of the brain AM. As noted, while pan-cytokeratin expression is observed in both layers of the arachnoid, expression of the epithelial membrane antigen was detected only at the level of the barrier cell layer (indicated by arrows

in Figs. 4C and 4D). The topographic distinction of the ABCL, as mentioned above, is related to the artifact formed during dehydration of the preparations, with the unstained area (marked with asterisks) located between the arachnoid barrier cell layer and the arachnoid reticular layer (Fig. 4C) and beneath the DM of the brain (Fig. 4D).

It is specifically noted that, under normal conditions, the absence of pan-cytokeratin (Clone AE1/AE3) and epithelial membrane antigen (Clone E29) activity in brain tissue is of particular importance for the differential diagnosis of epithelial metastases from glioblastomas within the brain.¹³

Considering that the ABCL is located between the CSF flowing in the interstitial and subarachnoid spaces of the DM, it is more appropriate to refer to the resulting biological barrier not as a blood-arachnoid barrier, but simply as an arachnoid barrier.¹⁴ The transport of tannic acid applied to the DM of the spinal cord in domestic ducks from the epidural space to the levels of the DM and AM is shown in Fig. 5A. As can be seen, in the spinal dura mater (indicated as SDM), the osmophilic deposits of tannic acid are clearly observed in the locations of collagen fibers surrounding the elastic fibers (marked EF) and fibrocytes (marked F), as well as in the surrounding ground substance. Although tannic acid molecules reached the level of the ABCL (indicated by wide arrows), no structures with osmophilicity differing from the control group were detected at the level of the arachnoid reticular cell layer's LMC (marked LMC) and collagen fiber bundles.

The data obtained at the electron microscopic level further confirm that the arachnoid barrier is located at the ABCL level. In addition, at the ABCL level, the thin osmophilic lines are located only between the cell plasmalemmas, which compared to other

¹³ Stoyanov, G.St., Dzhenkov, D., Ghenev, P. The great imitator – EMA positive glioblastoma multiforme // Scripta Scientifica Medica, – 2017. 49(1), – p. 12-16.

¹⁴ Smyth, LCD. Identification of direct connections between the dura and the brain / Leon C D Smyth, Di Xu, Serhat V Okar [et al.] // Nature, – 2024. 627(8002), – p. 165-173.

micromolecular tracers (lanthanum hydroxide and red ruthenium), indicates that tannic acid enhances the physical properties of the plasmalemma, preventing its entry into the cytoplasm of the cells. In the peripheral nerve bundles, within 1 hour of application, tannic acid spreads along with the epineurium to the level of the perineurial membrane, which serves as a biological barrier. While the collagen and elastic fibers in the inner layer of the epineurium, the perineurial cells in the outer two layers of the perineurium, and the collagen fibers between them are stained by tannic acid, no osmophilic deposits are observed in the basal laminae of the inner three layers of the perineurium, within the cell cytoplasm, or in the endoneurial space. The spread of tannic acid applied to peripheral nerve bundles over 2 hours is shown in Fig. 5B.

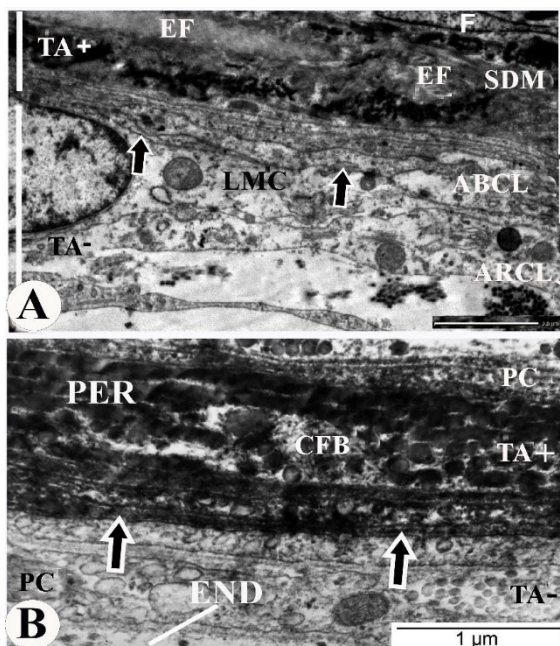


Figure 5. Electron micrographs of the transport of 1% tannic acid applied to the epidural surface of the duck's spinal cord meninges (A) and around the sciatic nerve of a white rat (B), taken after 2 hours.

As seen, all collagen fibers and most of the interstitial substance up to the regions of the perineurium marked with wide arrows are stained by tannic acid, and the latter is freely transported up to the inner layers of the perineurium (indicated as TA+), which can be stated without exaggeration. Moreover, the presented electron micrograph further confirms that the biological barrier responsible for maintaining the composition of the fluid in the endoneurial space is provided by the cells of the inner layer of the perineurium.

Additionally, the difference in density created by the microtracer around the inner layer of the perineurium may facilitate the transport of endoneurial fluid toward the epineural membrane.

In the control group, at the ABCL level of the white rats, arterioles entering the subarachnoid space branch out, forming a microcirculatory vascular network between the ARCL trabeculae, which includes precapillary arterioles, capillaries, postcapillary venules, and collecting venules. The collecting venules penetrate the ABCL and open into the DM venous vessels. These vessels differ from the DM vessels in two main features. First, all of them are surrounded externally by LMC. Second, the peripheral portions of the endothelial cells forming their inner layer are tightly connected, creating a blood–CSF barrier.

During experimental endotoxemia, the accumulation of cytoskeletal elements in the inner layer of arterioles led to the loss of integrity in the peripheral portions of endothelial cells, bulging of the plasmalemma into the vessel lumen, the formation of open intercellular spaces between adjacent endotheliocytes, a sharp increase in caveolae, and disruption of the three-layered structure of the internal elastic lamina. In capillaries and venular vessels, the first type of necrosis was observed, characterized by the accumulation of edema fluid between endothelial cells, loss of plasmalemma integrity, the formation of intercellular gaps, and in some cases, complete loss of the endothelial lining. The changes observed in the endothelium of all microvessels sharply increased vascular permeability, leading not only to deformation of the arachnoid membrane but also to disruption of its structural integrity. In the control group, at the AM level (Figure 6A),

noticeable unstained areas or deformation of cellular elements are not observed. In acute endotoxemia, at the light microscopic level, edematous fluid accumulates around both arterial (left in Figure 6B) and venous (right in Figure 6B) vessels located within the AM. In areas of lower resistance, this fluid compresses the membranous elements, resulting-compared with the control group-in the formation of extensive unstained areas (marked with an asterisk in Figure 6B).

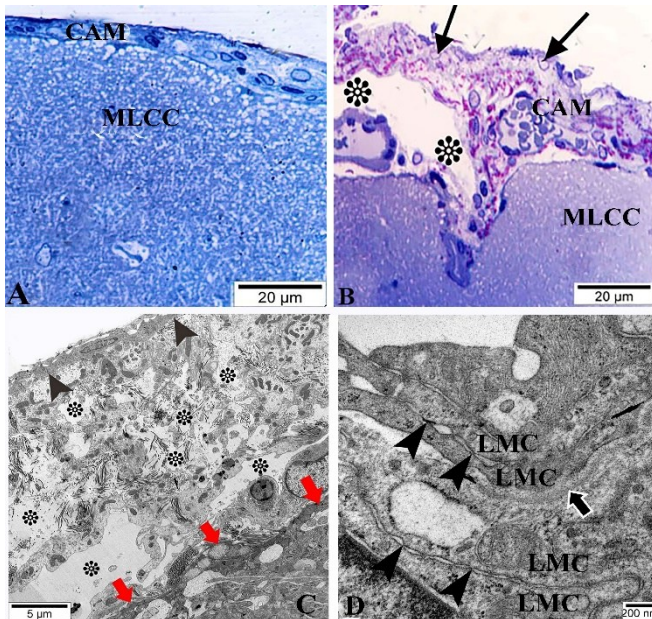


Figure 6. Light (B) and electron microscopic images (C and D) of changes in AM due to edema fluid exposure during endotoxemia compared to the control group (A). The explanation is given in the text. The abbreviations are shown in the images above.

At the same time, even at the light microscopic level, bundles of collagen fibers, distinguished by their fuchsinophilia, are detected throughout the AM, including in the intercellular spaces of cells that perform a barrier function. This indicates that, due to the loss of integrity of the ABCL (shown with arrows in Figure 6B), there is no

obstacle preventing the spread of edematous fluid and CSF from the subarachnoid space toward the dura mater. It should be noted that, under the influence of the edematous fluid (locations marked with an asterisk in Figure 6C), the pronounced deformation of the CFB, LMC and the microvessels, located between the ABCL (indicated with arrowheads) and the outer (surface) limiting glial membrane (shown with broad arrows), is clearly visible. As noted above (Figure 1A), in the control group, at the ABCL level, intercellular spaces of approximately 20 nm, located between the plasma membranes of the first and second layers of the LMC - typical for tight junctions-are not observed. Interestingly, during experimental edema, the tight junctions between the LMC plasma membranes (indicated with arrowheads in Figure 6D) and the presence of the 20 nm intercellular spaces between them are clearly evident. In the presented electron micrograph, as a result of the effect of edematous fluid, the cell layers involved in the organization of the ABCL are bent, leading to the cells of the barrier layer (indicated as LMCs) being positioned one above the other, both above and below the basal lamina (shown with a broad arrow), forming a two-tiered structure. Thus, in the control group, during fixation of the meninges, the extreme proximity of the first and second layers of the LMC at the ABCL level largely prevents accurate determination of the topographic arrangement of the junctions and intercellular spaces. These observations should be taken into account when studying the topography and ultrastructure of the barrier cell layer.

Comparative analysis of the obtained data indicates that the presence of differentiated macrophages, alongside monocytes, between the AM and leptomeningeal cells - both in the control group and during acute endotoxemia - demonstrates that these cells perform the function of a third line of defense at the level of the meninges. During acute endotoxemia, an increased number of macrophages is observed in regions where inflammatory agents may penetrate (in perivascular space of the spinal ganglia and in the subperineural spaces of the sciatic nerves), further confirming their participation in innate immune defense. A second important feature regarding macrophages

is that, during endotoxemia, they undergo pyroptosis, a form of programmed cell death.

Finally, it should be noted that, considering the destructive changes observed within just two hours of exposure to *E. coli* endotoxin are unlikely to be limited to the study period, special attention should be paid to preventing the formation of edematous fluid during inflammatory processes of various origins by regulating vascular permeability. Detailed investigation of the molecular mechanisms that prevent endothelial cell deficiency in acute endotoxemia could contribute to the development of effective strategies to counter these disturbances, as well as to the evaluation of new markers necessary for the early diagnosis of meningitis symptoms and their proper treatment.

RESULTS

1. Both in the central and peripheral nervous systems, the structures that serve as the first barrier line are formed from (the outside inward) the layers of leptomeningeal and perineural cells, which are in direct contact with the cerebrospinal and endoneural fluids, respectively. However, the second (subsequent) barrier – blood-CSF barrier – operating at the level of the pia mater and the reticular layer of the AM in the brain and spinal cord, is provided by the endothelial cells of blood vessels (regardless of the type of vessels) in the respective regions. In the control group, the presence of macrophages containing lipofuscin granules in their cytoplasm, alongside monocytes in the A and pia mater, indicates the formation of a third (immune) barrier [1, 2, 10, 13].

2. The existence of a cellular boundary layer of the DM has been confirmed both in mammals and birds. In mammals, the ABCL and ARL boundaries are formed by a discontinuous basal lamina or CFBs. In birds, within the ABCL and ARCL due to the extreme compaction of the plasma membranes of LMCs that support each other, along with the basal laminae located between them, dark osmiophilic structures are formed at the boundaries of the described layers. At the level of the ARL, the high number and dense arrangement of finger-like

projections make it impossible, even at the ultrastructural level, to delineate the borders of the LMCs [9, 10, 18, 20, 21].

3. Starting from the LMCs that form the arachnoid barrier, there is pronounced compaction of cytoskeletal elements, condensation of chromatin structures involved in nuclear organization, and in the surface-layer cells, the nucleus is often completely absent. Among the organelles, only occasional ribosomes and mitochondria in a state of structural disruption are observed. These changes indicate keratinization occurring in the surface layers of the AM [10, 14, 17, 19].

4. In all meningeal elements of the central and peripheral nervous systems, the integrity of the arachnoid and perineural barriers is disrupted in regions where arterial vessels enter and venous vessels exit, respectively. The origin of the spinal cord's denticulate ligaments, combined with the absence of structures capable of serving as barriers at the subarachnoid angles formed at the level of the nerve roots - where "CSF-conducting" channels are observed - indicates the possibility of cerebrospinal fluid being transported from the subarachnoid space toward the DM [4, 5, 7, 8]

5. The absence of detectable vimentin expression at the DM level is due to the fact that the perinuclear and peripheral regions of fibrocytes located there - which contain vimentin - have transverse dimensions ranging from 100 to 300 nm, making their detection physically impossible even under high magnification of a light microscope. While pan-cytokeratin expression is observed in both layers of the AM, expression of the Epithelial Membrane Antigen (EMA) is detected only at the level of LMCs involved in forming the arachnoid barrier [12, 18, 19].

6. As a micromolecular tracer, tannic acid applied to the epidural space (for 2 hours) freely passes through the dura mater elements and the surface layers of the AM to reach the plasma membranes of LMCs forming the arachnoid barrier. When applied around fragments of the sciatic nerve, tannic acid freely crosses the epineurial elements and the surface layers of the perineurium to reach its inner layer, which directly surrounds the endoneurial space and serves as a barrier. The formation of

a difference in concentration gradient of the microtracers between a single layer of LMC and perineural cell boundary can respectively, facilitate the transport of CSF toward the DM and endoneurial fluid toward the epineurium [2, 7, 15, 16].

7. During experimental edema, under the effect of LPS, structural changes occur in endothelial cells throughout the subarachnoid space-from arterioles to collecting venules. These changes include disruption of endothelial integrity, formation of transendothelial channels involving caveolae and micro- and macropinosomal vesicles, the emergence of open intercellular junctions, and in some areas, loss of the endothelial lining. As a result of the sharply increased vascular permeability and the impact of the resulting edematous fluid, the AM undergoes varying degrees of deformation and loss of integrity [3, 6, 8, 11].

PRACTICAL RECOMMENDATIONS

1.The factual material obtained through the application of complex methods used in the study allows for the identification of structures that serve as biological barriers in other parts of the organism.

2. The disruption of the integrity of the meningeal elements occurring within only 2 hours as a result of edema fluid formed due to a sharp increase in vascular permeability induced by intravenous administration of a low dose (1 mg/kg) of *E. coli* lipopolysaccharide may serve as a model for identifying the molecular mechanisms of processes that occur at the initial stages of the development of meningitis of various origins.

3. At the sites where the denticulate ligaments of the spinal cord originate from the dura mater, intrathecal injection of pharmacological agents and stem cells can be performed using minimally invasive techniques without disrupting the integrity of the arachnoid (meningeal) barrier.

4. The obtained micrographs, electronograms, and diagrams can be used in the teaching of human anatomy, pathological anatomy, neurology, cytology, embryology, and histology.

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ABBREVIATIONS

ABCL	– arachnoid barrier cell layer
AM	– arachnoid mater
ARCL	– arachnoid reticular cell layer
CAM	– cranial arachnoid matter
CDM	– cranial dura mater
CFB	– collagen fiber bundles
CSF	– cerebro spinal fluid
DBCL	– dural border cell layer
DM	– dura mater
EF	– elastic fibers
END	– endoneurium
F	– fibrocyte
LD	– lipid droplet
LMC	– leptomeningeal cell
LMCC	– cytoplasm of LMC
LMCPs	– processes of LMC
LPS	– lipopolysaccharide
MLCC	– molecular layer of cerebral cortex
PC	– perineural cell
PM	– pia mater
PER	– perineurium
SDM	– spinal dura mater
TA	– tannic acid



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