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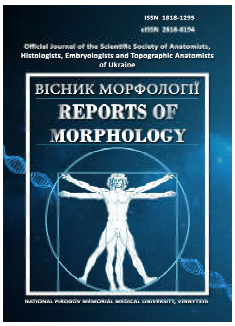

Histological structure of intercellular fluid circulation pathways

Kondor Yu. Yu., Tykholaz V. O., Guminskyi Yu. Yo.
National Pirogov Memorial Medical University, Vinnytsia, Ukraine

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CORRESPONDING AUTHOR
e-mail: kondorgeorg@gmail.com
Kondor Yu. Yu.

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Studies of the nervous system today are quite relevant and important. There are a large number of methods of studying and researching the brain, among which the histological method is widely used. Despite the variety of methods, in the possible practical application of histological examination of the central nervous system and brain in particular there are problems encountered by researchers: the complexity of the method, a large number of conventions to consider when working with nerve tissue, methods of fixation. Among other research methods, there is a group of histological methods, united by a common feature - in vivo staining of the nervous system, among which there is also a method of in vivo staining with methylene blue. The aim of the study was to establish the morphological features of the structure of the intercellular fluid circulation of the brain in experimental animals. The article describes a new method of injectable in vivo staining of the brains of laboratory animals with methylene blue. It is relevant for studying the morphology of the intercellular fluid circulation of the brain and the study of the structure of the microcirculatory tract. In our work it is offered to combine already known methods of perfusion fixation and a technique of supravital staining with methylene blue. Since most scientific studies of the brain use conventional research methods without a comprehensive study of the entire microcirculatory tract and intercellular fluid circulation, there is a need for more detailed study of the histological structure and topography of microcirculatory brain complexes to establish their normal structure. The results of the study confirm the researchers' observation that methylene blue has a high affinity for nerve fibers in the brain. In addition, it is obvious that the additional use of formalin as a solvent increases the resistance of methylene blue to leaching and the ability to stain the structures of the microcirculatory tract. In addition, the lifetime application of this technique allows you to visualize the morphological features of the microcirculation pathways of the intercellular fluid of the brain, Virchow-Robin space and capillary walls.

Keywords: *nervous system, glymphatic system, methylene blue, in vivo staining technique.*

Introduction

In modern science, the study of the nervous system has a special place. In recent decades, research on the brain, nervous tissue, and neurophysiology has advanced significantly. However, together with progressive studies of the properties of proteins, gene expression and the effects of neurotransmitters, morphohistological methods of studying the nervous system are still relevant.

To study the structure of nervous tissue, there are methods that are used to satisfy certain tasks set by the researcher. Despite the variety of methods, in the possible practical application of histological examination of the central nervous system in general and the brain in particular, there are problems encountered by researchers: the complexity of the method, the large number of conventions to consider when working with nerve tissue, methods of fixation [15].

Among the known methods of histological examination, which are recommended for work with nervous tissue, are: the method of staining with toluidine blue (blue) according to Nissl, the "gold standard" of histology - staining with hematoxylin-eosin and methods of impregnation [15]. The above techniques have one characteristic feature - they are associated with long-term fixation with formalin, alcohol and xylene, which are certainly necessary measures to preserve the samples. Among other research methods, there is a group of histological methods, united by a

common feature - in vivo staining of the nervous system, among which is a method of in vivo staining with methylene blue.

The method of using methylene blue in vivo is not new, its use was described by Ehrlich in 1886, and the method was improved and refined by Dogiel and Cajal (1896-97) [14]. The advantages of this technique are the staining of still living tissues, especially when it comes to a vital way of staining. According to Ehrlich- Dogiel, a sequence of actions has been formulated to ensure the optimal staining of cells of the nervous system, which consists in the lifelong introduction or shortly after euthanasia into the bloodstream or directly into the parenchyma of the organ. The peculiarity is that this method of staining is used not only in the study of the central nervous system, but more to detect neurons, neuroglia, nerve endings and axons of the peripheral nervous system, which is considered the primary goal of the method and has more advantages than CNS [14]. In addition, the substance used for staining is selectively specific in three respects: first, methylene blue is a specific dye for cells of the nervous system and is relatively non-toxic to cells of the nervous system; secondly, as a nitrogen-containing compound, it reacts with nitric oxide, which leads to a heterogeneous distribution of the dye in the tissue due to the fact that living cells break down methylene blue, and non-living - accumulate dye; third, methylene blue is a substance that crosses the blood-brain barrier. The above-mentioned phenomena are a consequence of the chemical structure and properties of both the substance and the biochemistry of cells [11]. This question remains open today [14].

At the electron microscopic level in supravital research, researchers have developed a method of visualization of dye accumulation sites in neurons. However, their techniques include complex fixation after brain sampling, when after sampling the stained organ requires additional fixation with special chemical compounds (eg ammonium picrate, osmium compounds, molybdenum). These compounds are primarily toxic and are used to prevent methylene blue leaching during fixation in aldehydes or alcohols [11, 14, 15].

When studying the structure of the brain, scientists widely use the method of perfusion fixation. Given the size of the samples, the purpose of fixation is to quickly and evenly preserve the tissue in an unchanged state [8, 9]. Often, changes in response to hypoxia begin before the tissue can be preserved [17]. The advantage of direct fixation through the circulatory system is that the chemical can quickly reach all parts of the body through the natural vascular network. Using this method of fixation, it is necessary to take into account the parameters of the circulatory system, such as pressure, pH, temperature, which significantly improves the result [6]. The main advantage of this method (compared to volumetric methods) is that the circulatory system is used most efficiently [4].

In our work it is offered to combine already known methods of perfusion fixation and a technique of supravital staining with methylene blue. Since most scientific studies of the brain use conventional research methods without a comprehensive study of the entire microcirculatory tract and intercellular fluid circulation, there is a need for more detailed study of the histological structure and topography of microcirculatory brain complexes to establish their normal structure.

The aim of the study was to establish the morphological features of the structure of the intercellular fluid circulation of the brain in experimental animals.

Materials and methods

Humane principles of animal care were applied in the work, and all procedures complied with the current law on animal protection.

The following methods were used: methylene blue (pure for analysis), previously diluted to a concentration of 1 % (1 g per 100 ml) with 10 % formalin solution in phosphate buffer (pH=7.35) and filtered before use; heparin; 0.9 % sodium chloride for exsanguination; 5 % glucose to prevent gliolysis; systems for intra-arterial infusion; G26 Vasafix catheters; sodium thiopental for anesthesia; sucrose solutions 15 % and 30 % for cryoprotection.

5 male Oryctolagus cuniculus rabbits weighing 1 to 1.5 kg were selected. The animals were kept in good condition with free access to food and water. All conditions of detention and all procedures with animals were carried out in compliance with bioethics (Protocol of the **Commission on Bioethics** № 2 of 09.03.2021) and in compliance with the ethical principles of the European Convention for the Protection of Vertebrate Animals.

Animals were anesthetized with sodium thiopental (1 mg/kg administered intraperitoneally), and the common carotid artery was isolated and catheterized with a Vasafix G26 catheter. The vascular bed was bled and washed with 0.9 % sodium chloride solution up to 4 ml. To prevent gliosis, up to 1 ml of 5 % glucose solution was administered. After rinsing the vascular bed with a syringe, a system with a vial of 1 % filtered solution of methylene blue in 10 % buffer solution of formalin preheated (t =37°C, pH=7.34-7.45, V=15-20 ml) was connected to common carotid artery drip (60 drops/min). Adjustment of the solution injection pressure was provided by raising the vial on a tripod to a height of 100-120 cm, which corresponded to the design pressure of 120 mm Hg. Methylene blue was continued until the animal's skin and mucous membranes turned blue. Euthanasia of the animal was performed by overdose of sodium thiopental.

The brain was isolated and removed by decapitation and dissection of the skull vault. After removal, the brain sample was immersed in a cooled buffer solution of 10 % formalin (t =4°C, pH=7.34-7.45) for one day, followed by cryoprotection in 15 % and 30 % buffer solutions of sucrose (chemically pure) in each solution at t =0-4°C for two days.

After pre-fixation and cryoprotection, sections 10-15 μm thick were made using a freezing microtome (t =-15-20 C). Subsequently, micropreparations made on slides were examined under a light microscope (SCOPE S/N EU1960762). Surveys were performed using a sCMEX Euromex Microscopen B.V. DC.1359 F100.

Results

Macroscopic examination of brain samples reveals a change in tissue color from pale pink to blue. Contrast-filled vessels are well traced: vessels of the circle of Willis, middle cerebral arteries, ocular artery (Fig. 1). The meninges have acquired a stable blue color. The stem tissue of the brain is well stained. In the cross-section of the brain there is a clear demarcation of gray, white matter, nuclei and conductive pathways of the brain. With a more saturated shade of blue, the gray matter is clearly visualized (Fig. 2, 3).

Capillaries with erythrocytes were well visualized on micropreparations of cryosection of rabbit brain 10-15 m in the frontal and horizontal planes. The capillary wall is painted blue, around which the so-called Virchow-Robin space is clearly visible, bounded on the outside by a layer of glia cells, painted in deep blue [1]. The accumulation of methylene blue contrast accompanies capillaries and clusters of glia, located in the form of tangentially directed "strands", presumably along the paraaxonal outflow path of the intercellular fluid of the brain (see Fig. 2, 3). The above picture is heterogeneous and depends primarily on the layer of bark, the direction of axon fibers in the white matter, the location of the capillary network, radial glia, and so on. This pattern can be well seen in the direction from the meninges, where the dye enters the brain parenchyma with capillaries, accumulates in the gray matter, where the capillaries, neuroglia accumulation or axon location spreads to the white matter, reaching the basal nuclei of the brain. to other structures (Fig. 2, 3, 4, 5).

This type of distribution of methylene blue in brain tissue can be seen in the comparison of sections of different thickness and when changing the plane of the sections

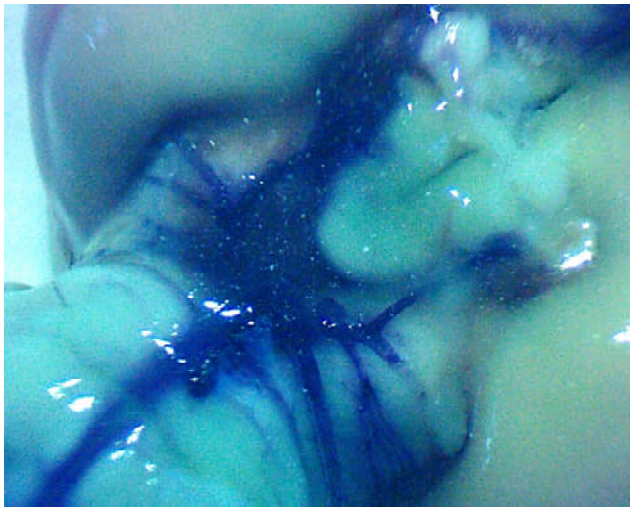

Fig. 1. The vessels of the rabbit's basis cerebri are filled with methylene blue. The change in color of a parenchyma of a brain is well visible.

Fig. 2. Morphology of paravasal spaces of the cerebral cortex using light microscopy. Horizontal section of the rabbit brain. Method of lifelong staining with methylene blue. x10.

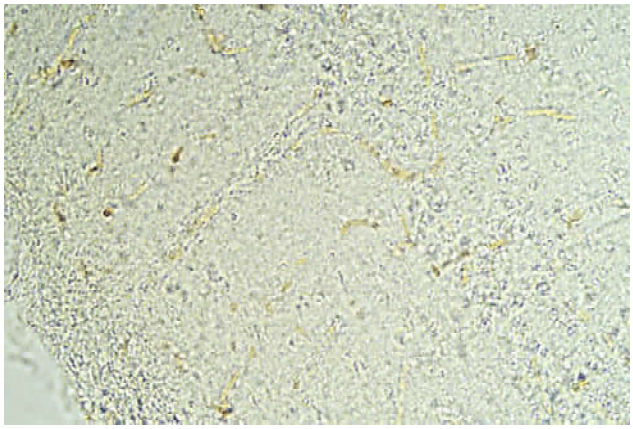

Fig. 3. The structure of the microcirculatory tract, glia cells, paravasal spaces, cerebral cortex. Method of lifelong staining with methylene blue. x40, 10-15 μm.

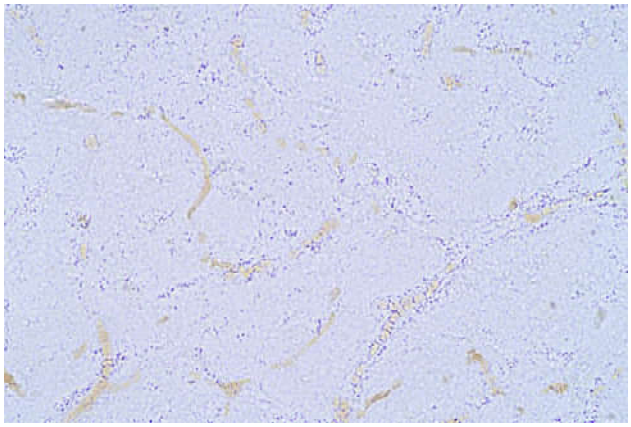

Fig. 4. The structure of the microcirculatory tract, the white matter. Method of lifelong staining with methylene blue. x10, 10-15 μm.

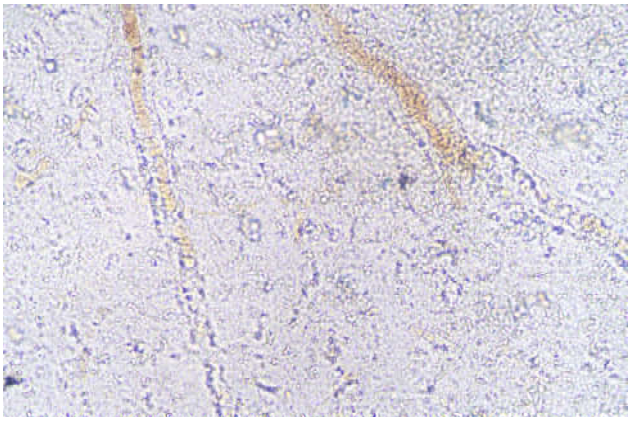

Fig. 5. The structure of the microcirculatory tract, the white matter. Method of lifelong staining with methylene blue. x40, 10-15 μm.

from frontal to horizontal (Fig. 4, 5).

In contrast to the well-known Nissl method, in this case the neurons were not stained, as methylene blue accumulated only in neuroglia cells, capillary endotheliocytes and intercellular space. We assume that this occurs due to the biochemical properties of methylene blue and the conditions of lifelong staining [11].

Discussion

This method of in vivo injection of intra-arterial staining and fixation of the brain with methylene blue provides a better study of the microcirculatory tract and the circulation of intercellular fluid of the brain [10].

The morphological characteristics of the studied material primarily describe the distribution of methylene blue in brain tissue. The latter repeats the path of intercellular fluid circulation: from the capillaries to the Virchow-Robin space, then into the intercellular space by transglial and paraaxonal pathways towards the venous system [1, 2]. This pathway of intercellular fluid circulation by some authors, such as Maiken Nedergaard [7, 10], is defined as a system of purification and regulation of fluid balance in the brain and is called "glymphatic system" [13].

First of all, it should be noted that the main substance in this technique, methylene blue, is commonly used as an inhibitor of cytosolic guanylyl cyclase, a physiological receptor for nitric oxide [5, 11, 14].

Methylene blue is a thiazide dye that selectively stains the nuclei of neurons, endothelium, glia, axons, and others. In addition, due to the interaction with nitric oxide receptors, blocking them, it does not stain living neurons in the brain, selectively staining neuroglia, meninges and capillary wall. Although Cajal (1897) described the ability of methylene blue to stain perineuronal networks around neurons [3, 14].

In contrast to the above characteristics of the aqueous solution of methylene blue, we used a solution of formalin 10 % in buffer solution. The main physicochemical characteristics of the microcirculatory tract were also taken into account, in particular: pressure, temperature, pH, etc.

All the above actions for the methylene blue solution are explained by the following observations. Changing the base for the methylene blue solution slightly increased the resistance of methylene blue to leaching and allowed simultaneous fixation and staining while the nerve tissue remained alive. Also taken into account the characteristics of the solution were close to the characteristics of the microcirculatory tract (t =37°C, pH=7.34-7.45, V=5-10 ml, P=100/120 mm Hg), which in turn reduced the negative impact on the nervous tissue.

It should also be noted that the use in this study of the formalin base for the solution is considered a valid option for its use as a fixative, because there are similar methods of fixing the brain in rodents. The latter is widely used in immunohistochemical studies and electron microscopy [4, 12, 16].

Further studies are planned to link the supravital use of methylene blue directly to biochemical and electrophysiological experiments; which would give more insights into the extra- and intracellular metabolic pathways of the dye. It is also planned to supplement the method with other substances specific to the nervous system, and to apply the method of injectable in vivo staining of the brains of laboratory animals with methylene blue for other organs and systems.

Conclusions

1. The results of the study confirm the observations of Ehrlich and other researchers that methylene blue has a high affinity for nerve fibers in the brain. It is obvious that the additional use of formalin as a solvent increases the resistance of methylene blue to leaching and the ability to stain the structures of the microcirculatory tract. Lifetime application of this technique allows to visualize the morphological features of the microcirculation pathways of the intercellular fluid of the brain, Virchow-Robin space and capillary walls. Therefore, the demonstrated fixation technique should be considered a significant improvement of the modern method.

2. Due to its simplicity and high specificity to nervous tissue, the described technique is a useful addition to other histological methods, especially those that visualize other types of nerve fibers, such as myelin staining methods.

3. Due to the ability to visualize the morphological features of the microcirculation pathways of the intercellular fluid of the brain, this method is a useful addition to neuroanatomy.

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ГІСТОЛОГІЧНА СТРУКТУРА ШЛЯХІВ ЦИРКУЛЯЦІЇ МІЖКЛІТИННОЇ РІДИНИ

Кондор Ю. Ю., Тихолаз В. О., Гумінський Ю. Й.

Дослідження нервової системи на сьогоднішній день є досить актуальними і важливими. Існує велика кількість методів вивчення та дослідження головного мозку, серед яких широко використовується гістологічний метод. Попри різноманіття методик, у можливому практичному застосуванні гістологічного дослідження центральної нервової системи та головного мозку зокрема виникають проблеми, з якими зустрічаються дослідники: складність виконання методики, велика кількість умовностей, які потрібно враховувати при роботі з нервовою тканиною, способи фіксації. Серед інших методів дослідження виокремлюється група методів гістологічного дослідження, об'єднаних спільною рисою - прижиттєве забарвлення нервової системи, серед яких до того ж є спосіб прижиттєвого забарвлення метиленовим синім. Метою дослідження було встановлення морфологічних особливостей будови шляхів циркуляції міжклітинної рідини головного мозку експериментальних тварин. У статті описана нова методика ін'єкційного прижиттєвого забарвлення головного мозку лабораторних тварин метиленовим синім. Вона є актуальною для вивчення морфології шляхів циркуляції міжклітинної рідини головного мозку та дослідження структури мікроциркуляторного русла. У нашій роботі пропонується поєднати уже відомі методи перфузійної фіксації та методику суправітального забарвлення метиленовим синім. Оскільки більшість наукових праць, в яких досліджували головний мозок, використовують звичайні методи дослідження без комплексного дослідження всього мікроциркуляторного русла та шляхів циркуляції міжклітинної рідини, то виникає потреба у більш детальному дослідженні гістологічної структури та топографії мікроциркуляторних комплексів головного мозку для встановлення їх нормальної будови. Отримані результати дослідження підтверджують спостереження дослідників про те, що метиленовий синій має високу спорідненість до нервових волокон головного мозку. Крім того, очевидно, що додаткове застосування формаліну, як розчинника, підвищує стійкість метиленового синього до вимивання та здатність до забарвлення структур мікроциркуляторного русла. До того ж, прижиттєве застосування даної методики дозволяє візуалізувати морфологічні особливості шляхів мікроциркуляції міжклітинної рідини головного мозку, простори Вірхова-Робінсона та стінки капілярів.

Ключові слова: *нервова система, глімфатична система, метиленовий синій, прижиттєва методика забарвлення.*