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SMI-32 — A NOVEL AXONAL INJURY MARKER FOR INVESTIGATION OF ISCHEMIC BRAIN PATHOLOGY

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The relevance of this work is determined by the high prevalence and social significance of cerebrovascular diseases and the need to develop effective methods for verifying neuronal damage due to cerebral ischemia in experimental models.

The aim of this study was to assess the possibility of immunohistochemical revealing of neurofilaments to detect axonal injury in cerebral ischemia models.

Materials and methods. A model of transient focal cerebral ischemia by the left middle cerebral artery occlusion was reproduced in male Wistar, SHR and WKY rats. Axonal injury was assessed by immunohistochemical reactions for neurofilament proteins using SMI-32 and 2F11 antibodies.

Results. In cerebral ischemia, damage to nerve fibers occurs, manifested by thickening of axons, their varicose expansion and segmental accumulation of neurofilament proteins. These changes are more noticeable with an immunohistochemical reaction to the SMI-32 marker of neurofilament heavy chain.

Conclusions. The use of antibodies to the non-phosphorylated neurofilament heavy chain makes it easy to identify degenerating nerve fibers and can be recommended as an alternative method for detecting axonal injury.

Keywords: SMI-32; axonal injury; cerebral ischemia.

SMI-32 КАК МАРКЕР АКСОНАЛЬНОГО ПОВРЕЖДЕНИЯ ПРИ ИШЕМИИ ГОЛОВНОГО МОЗГА

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Актуальность данной работы определяется высокой распространенностью и социальной значимостью цереброваскулярных заболеваний и необходимостью разработки эффективных методов верификации нейронального повреждения вследствие ишемии головного мозга в экспериментальных моделях.

Целью настоящего исследования стала оценка возможности использования иммуногистохимической реакции на белки нейрофиламентов для выявления аксонального повреждения при моделировании ишемии головного мозга.

Материалы и методы. На крысах-самцах линий Вистар, SHR и WKY была воспроизведена модель фокальной транзиторной ишемии головного мозга путем окклюзии левой средней мозговой артерии. Повреждение аксонов оценивали с помощью иммуногистохимических реакций на белки нейрофиламентов, используя антитела SMI-32 и 2F11.

Результаты. При ишемии головного мозга происходит повреждение нервных волокон, проявляющееся утолщением аксонов, их варикозным расширением и сегментарным накоплением белков нейрофиламентов, причем данные изменения более заметны при иммуногистохимической реакции на маркер тяжелых цепей нейрофиламентов SMI-32.

Заключение. Использование антител к нефосфорилированной форме тяжелых цепей нейрофиламентов позволяет легко идентифицировать дегенерирующие нервные волокна и может быть рекомендовано в качестве альтернативного метода выявления аксонального повреждения.

Ключевые слова: SMI-32; аксональное повреждение; ишемия головного мозга.

Problems on the prevention and treatment of cerebrovascular diseases have been addressed because of the high prevalence of cerebral lesions of vascular origin [1]. Easily reproducible adequate experimental models of cerebral ischemia have been successfully developed and designed to test the effectiveness of new preventive and therapeutic approaches. However, further progress in this direction is hindered by the lack of simple and informative methods for assessing neuronal

and gliocyte damage. One of the methods for detecting dying neurons is the immunohistochemical detection of the protein NeuN in the nuclei of nerve cells [2]. Despite the detection of this neural marker, it cannot be used to assess the state of nerve fibers. On the contrary, the Marchi classic impregnation technique can be applied to detect the degenerative changes in the myelin sheaths of nerve fibers. In this method, parts of the brain are treated with salts of heavy metals and osmic acid for several weeks, thereby staining a degenerating myelin [3, 4]. Although the quality of the obtained preparations is high, the application of the Marchi method in laboratory practice becomes complicated because of the duration of material processing and the use of highly toxic substances.

One of the modern methods suitable for assessing the state of nerve fibers is the immunohistochemical detection of various cytoskeletal markers. Among various markers, neurofilament proteins are the most specific for neuronal processes. Neurofilaments are intermediate filaments that compose the cytoskeleton of nerve cells. They consist of several proteins (chains): light NF-L (68 kDa), intermediate mass NF-M, and heavy NF-H (200 kDa) [5]. For example, internexin is a neurofilament protein that participates in the formation of a quaternary structure. In addition to structural characteristics, the phosphorylation of neurofilament chains should be examined because it reflects their functional activity to some extent. In the present study, the antibodies of the unphosphorylated heavy chains of clone SMI-32 and the antibodies of the phosphorylated light chains 2F11 widely used in scientific research were selected to detect neurofilament proteins [6].

This study aimed to determine the possibility of carrying out an immunohistochemical reaction involving neurofilament proteins to identify pathological changes in nerve fibers in the modeling of cerebral ischemia.

Materials and methods

Experiments were performed on an archival material of mature male normotensive Wistar ($n = 5$) and Wistar Kyoto (WKY, $n = 5$) rats and the spontaneously hypertensive rats (SHR, $n = 11$) from previous experiments [7, 8]. The care and killing of animals were performed in accordance with the Rules for Conducting Work with the Use of Experimental Animals (annex to the order of the USSR Ministry of Health No. 755 of 08/12/1977), the Helsinki Declaration of 1975, and its revised version in 2000. This study was approved by the local ethics committee of Experimental Medicine Institute (protocol 3/19 dated 04/25/2019). Ischemia was modeled with the endovascular occlusion of the left middle cerebral artery under general anesthesia. Circulatory disturbances in the basin of the left middle cerebral artery in each case were induced for 30 min, and reperfusion time was set to 48 h. The corresponding structures of the telencephalon of the intact SHR ($n = 3$), WKY ($n = 3$), and Wistar ($n = 5$) rats. The brain was fixed in a zinc–ethanol–formaldehyde solution, dehydrated, and embedded in paraffin in accordance with the standard techniques. The target brain of experimental and intact rats was used as an object for morphological analysis, given that ischemia was modeled with the occlusion of the middle cerebral artery, which supplies the striatum, hippocampus, and temporal lobe of the cerebral hemispheres. Serial frontal sections of the 5 μm-thick telencephalon prepared on a Leica RM2125RT rotary microtome (Leica, Germany) were morphologically examined. The sections were dewaxed, rehydrated, thermally unmasked in a freshly prepared 10% sodium thiosulfate solution, and subjected to immunocytochemical studies [9].

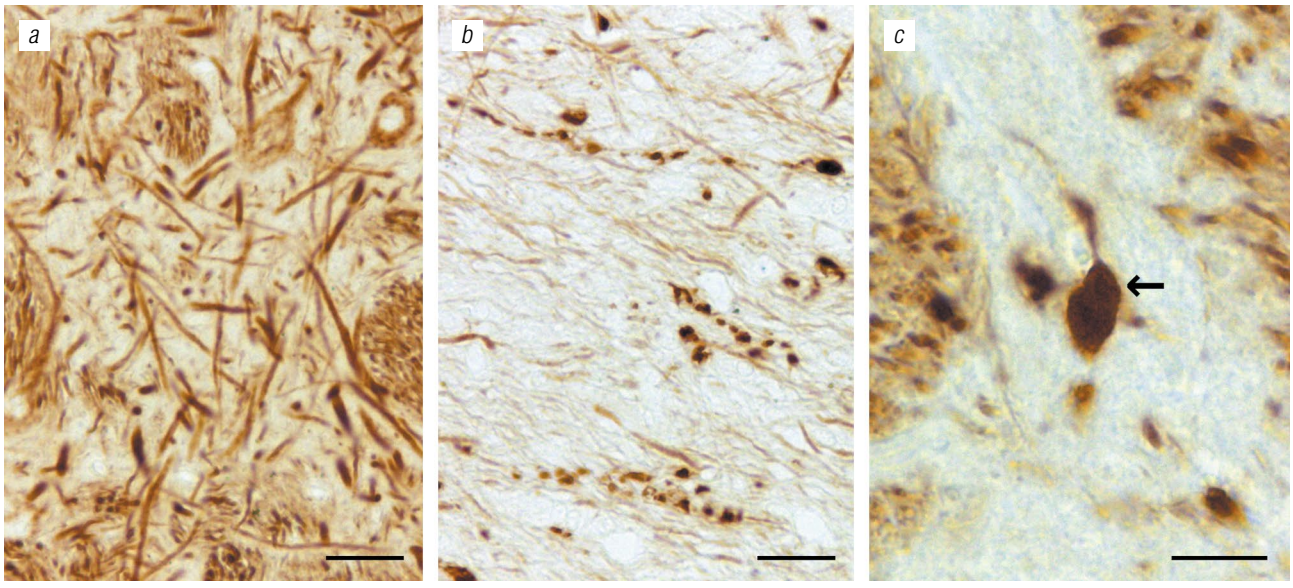
The murine monoclonal antibodies of unphosphorylated heavy (200 kDa) subunits, clone SMI-32 (dilution 1 : 1000; BioLegend, USA), and the murine monoclonal antibodies of phosphorylated light (68 kDa) subunits, clone 2F11 (dilution 1 : 300, Dako, Denmark), were used for the immunocytochemical detection of neurofilament proteins. Primary antibodies were detected using a Reveal Polyvalent HRP DAB Detection System kit (Spring Bioscience, USA). The peroxidase label was determined using a diaminobenzidine chromogen (DAB Quanto; Thermo Scientific, USA). After immunocytochemical reactions, some of the sections were stained with hematoxylin. A survey staining with cresyl violet was conducted on the basis of Nissl and an immunocytochemical reaction for NeuN to verify the ischemic damage to the experimental animals [7].

The preparations for microscopy in transmitted light were analyzed using a Leica DM750 microscope, and photographs were captured using an ICC50 camera (Leica, Germany).

Results

No signs of nervous tissue dystrophy and degeneration were found in the striatum and cerebral cortex of the intact SHR and WKY animals. During the immunocytochemical reaction on SMI-32, large axons with a diameter of 1.1–1.8 μm were most clearly detected in the white matter of the striatum, corpus callosum, and pallidum (Fig. 1, *a*). They were stained evenly throughout the entire length and had no thickening formation. The staining intensity was uniform among all the observed axons in the studied regions of the telencephalon. The perikarya of predominantly large neurons of the striatum and cerebral cortex were stained in the same way. The coloration of the perikarya appeared less uniform coarse grained, whereas the nuclei of the nerve cells were not stained. When the antibodies of clone 2F11 were used, the axons were also uniformly stained, but the perikarya of the neurons were not stained. The morphological and immunohistochemical characteristics of the neurons of intact Wistar rats did not differ from previous findings [10].

The foci of ischemic damage were observed in all the studied animals 48 h after 30 min of unilateral ischemia in the striatum and cortex of the left hemisphere. In the damaged area during the immunocytochemical reaction with SMI-32, the axons were hyperchromic and thicker (from 1.5 μm to 2.3 μm) than those in similar areas in intact animals; in some cases, the fragmentary staining of the axons was uneven as indicated by the chain type observed (Fig. 1, *b*). Numerous rounded and oval hyperchromic structures were clearly defined in the striatum and the V–VI layers of the cortex of the ischemic hemisphere at low magnification, and exceeded the size of the transverse sections of ordinary axons. The identified structures had no unstained central region. This finding indicated that they were unlikely to be small immunopositive neuronal perikarya. At high magnification, these structures could be characterized as varicose axonal sections because most of them looked like fusiform thickenings connected by thin sections of the cytoplasm (Fig. 1, *c*). These changes affected most of the



Осевые цилиндры нервных волокон в головном мозге крысы: *a* — контрольная крыса линии SHR (стриатум); *b, c* — крысы линии SHR через 48 ч после 30-минутной окклюзии средней мозговой артерии (*b* — таламус, *c* — стриатум). Стрелкой указан варикозно расширенный участок аксона. Иммуноцитохимическая реакция на нефосфорилированные белки нейрофиламентов (тяжелые цепи) с использованием антител SMI-32, визуализация с помощью хромогена диаминобензидина без подкраски. Масштабный отрезок равен 20 мкм (*a, b*) и 10 мкм (*c*)

Axial cylinders of nerve fibers in the rat brain: *a* — control SHR rat (striatum); *b, c* — SHR rats 48 h after 30 min of middle cerebral artery occlusion (*b* — thalamus, *c* — striatum). Arrow shows the varicose expansion of the axon segment. Immunocytochemical reaction for the unphosphorylated neurofilament heavy chain using SMI-32 antibodies and visualization with diaminobenzidine chromogen without counterstaining. Scales are 20 (*a, b*) and 10 μm (*c*)

axons located in the foci of ischemic damage to the striatum and cortex of the ipsilateral hemisphere. In the contralateral hemisphere, the axons in the striatum appeared normal, and no extensions were found.

When the antibodies of clone 2F11 were used at low magnification, drop-shaped structures were not detected. At high magnification, similar varicose axons could be identified, but they were not hyperchromic in comparison with the surrounding axons.

Discussion

The comparison of the early disappearance of NeuN from the perikarya of striatal neurons after short-term transient cerebral ischemia, discovered by us in previous studies [7]. In our study, the identified changes indicated that damage to neurons at the perikaryon level was accompanied with degenerative changes in the part of their axons. Such degenerative changes were manifested in the formation of pathological varices, the fragmentation of nerve fibers, and the pathological aggregation of unphosphorylated heavy subunits of neurofilaments. The neurofilament proteins of phosphorylated light chains did not accumulate. However, when neurons and their processes were damaged, the amount of synthesized neurofilament proteins could increase. The immunoreactivity of pathologically altered axons was high likely because of a double effect, namely, abnormal aggregation and dephosphorylation of neurofilament proteins. Therefore, SMI-32 antibodies obtained against the unphosphorylated form of neurofilament proteins were more effective than other antibodies in visualizing damage to nerve fibers.

With the presented methodological approach, pathological changes in nerve fibers formed by long-axon neurons can be identified. This approach is not unique, but it has a number of advantages over previously used techniques, including simple setup of reactions and clear visualization of pathological structures. Classical methods of detecting myelin degeneration include the Marchi method, which was proposed back in the 19th century. Both underwent a number of modifications [4]. The Kluver–Barrera method [11] is unreliable and even requires the special fixation of materials. The first of them involves the use of large volumes of osmic acid, which is an expensive reagent with volatility and high toxicity.

Some of the modern methods for assessing the state of myelinated nerve fibers are the detection of myelin components (such as myelin basic protein) and others [12–15]. However, they are not entirely suitable for studying axons in the central nervous system because they cannot be used to trace the course of individual nerve fibers. The method for detecting amyloid precursor protein (APP) accumulating in damaged axons is less widespread [16]. However, the reasons for the accumulation of whey protein in axonal degeneration have yet to be fully elucidated; consequently, the interpretation of results becomes complicated. The antibodies of myelin and APP may exhibit species specificity; as such, the same methods cannot be applied to detect axonal degeneration in disease modeling and diagnosis in humans. Nevertheless, the antibodies of clone SMI-32 may be utilized to explore the nervous tissues of humans and various animal species [6, 17–21].

Conclusions

As a result of short-term cerebral ischemia, neurodegenerative changes occur in the ischemic hemisphere of rats. These changes are accompanied with the accumulation of unphosphorylated neurofilament proteins in pathologically altered nerve fibers. The antibodies of the unphosphorylated form of heavy chains of neurofilaments may be used to identify degenerating nerve fibers and can be recommended as an alternative method for detecting axonal damage.

Additional information

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Compliance with ethical standards. The study was approved by the local ethics committee of the Institute of Experimental Medicine (protocol 3/19 dated April 25, 2019).

Conflict of interest. The authors declare no conflict of interest.

Authors' contributions

D.L. Tsyba — immunohistochemical reactions, analysis of the results, and writing of the text

O.V. Kirik —analysis of the results and writing of the text

D.E. Korzhevskii — general guidance and editing of the text.

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