DOI 10.26724/2079-8334-2022-3-81-252-256
UDC 616-071+616.833.5+616-089+615.849.19

Z.M. Yashchyshyn, S.V. Ziablitsev[1], L.M. Zaiats
Ivano-Frankivsk National Medical University, Ivano-Frankivsk
[1]Bogomolets National Medical University, Kyiv

IMMUNOHISTOCHEMICAL DETECTION OF NEUROFILAMENTS IN THE SCIATIC NERVE, WHICH REGENERATES AFTER NEUROTOMY AND SURGICAL SUTURING

e-mail: Zinoviy1964@i.ua

The purpose of the study was to determine the expression of neurofilaments in the sciatic nerve, which regenerates after neurotomy and surgical suturing, and to compare the effect of laser irradiation of different spectrum. The experiment was performed on 20 laboratory Wistar rats (200–250 g) with crossing the left sciatic nerve and suturing with an epineural suture end to end 30 minutes after neurotomy. 90 days later, an immunohistochemical study was performed using monoclonal anti-neurofilament antibodies (clone 2F11; Thermo Fisher Scientific; USA). In the control group (without the use of a laser), the main type of regenerated nerve fiber was a small diameter myelinated fiber (2–3 μm), the number and density of the myelin sheath of axons were low, and the growth of connective tissue was high. Neurofilaments expression is weak (up to 25 % of neurons) or absent. The use of laser irradiation of the green spectrum (560 and 520 nm) allowed to significantly increase the level of neurofilaments expression, and the use of irradiation of the blue spectrum (450–480 nm) – to restore the structure of the sciatic nerve.

Key words: nerve regeneration, neurofilaments, expression, immunohistochemistry

З.М. Ящишин, С.В. Зябліцев, Л.М. Заяць

ІМУНОГІСТОХІМІЧНЕ ВИЯВЛЕННЯ НЕЙРОФІЛАМЕНТІВ У СІДНИЧНОМУ НЕРВІ, ЩО РЕГЕНЕРУЄ ПІСЛЯ НЕЙРОТОМІЇ ТА ХІРУРГІЧНОГО УШИВАННЯ

Метою роботи було визначити експресію нейрофіламентів у сідничному нерві, який регенерує після нейротомії та хірургічного ушивання, та порівняння ефекту лазерного опромінення різного спектру. Експеримент проведено на 20 лабораторних щурах лінії Wistar (200–250 г) з пересіченням лівого сідничного нерва та ушиванням епіневральним швом кінець в кінець через 30 хвилин після нейротомії. Через 90 днів було проведено імуногістохімічне дослідження з використанням моноклональних анти-нейрофіламентних антитіл (клон 2F11; Thermo Fisher Scientific; США). У контрольній групі (без застосування лазера) основним типом регенерованого нервового волокна було міелінізоване волокно малого діаметра (2–3 мкм), кількість і щільність мієлінової оболонки аксонів були низькими, а розростання сполучної тканини – високим. Експресія NEFL виражена слабо (до 25 % нейронів) або відсутня. Застосування лазерного опромінення зеленого спектру (560 та 520 нм) дозволило суттєво збільшити рівень експресії нейрофіламентів, а застосування опромінення синього спектру (450–480 нм) – відновити структуру сідничного нерва.

Ключові слова: регенерація нерва, нейрофіламенти, експресія, імуногістохімія.

The work is a fragment of the research project "Pathogenetic mechanisms of changes development in the respiratory, endocrine, nervous system in the simulated pathological states and their correction", state registration No. 0117U001758.

Despite a significant progress in understanding the pathophysiology of peripheral nervous system damage and regeneration as well as achievements in the microsurgical techniques, peripheral nerve damage is a major problem for reconstructive surgeons [1, 2]. Better restoration of nerve structure and function largely depends on the introduction of discoveries in molecular biology and non-drug methods of physical rehabilitation [3, 8]. Peripheral nerve damage causes a sequence of degeneration and regeneration events with a specific time course, resulting in complete or partial functional recovery [10]. Immediately after damage to nerve integrity, the stage of early axon degradation begins, which passes into the stage of rapid degeneration, followed by germination and a long period of remyelination.

In the process of myelin regeneration, the main role belongs to neurofilaments [6]. Neurofilaments (NEFL) are found directly in the neurons and their branchings and consist mainly of three main proteins that differ in molecular weight – light (NEFL), medium (NEFM) and heavy (NEFH). NEFL is a low-molecular-weight basic structural subunit of microfilament with a molecular weight of approximately 70 kDa. Gene knockout Nefl in mice leads to a decrease in mRNA gene expression and NEFL protein content, which reproduces the symptoms of amyotrophic lateral sclerosis [6]. In addition, neurofilaments are involved in the molecular pathology of numerous neurodegenerative diseases, for example, axonal and demyelinating forms of Charcot-Marie-Tutu disease (CMT1 and CMT2) [9]. The NEFL content is significantly increased in the cerebrospinal fluid and serum in many neurological conditions, which makes it possible to use this index as a biomarker of nervous regeneration [5].

Previously, we studied the morpho-functional state of the structural components of the rat sciatic nerve after its complete intersection with end-to-end suturing with an epineural suture [2]. It was found that the use of monochromatic low-frequency laser irradiation with a Spektr-LC LED laser significantly reduced the recovery time, structure and function of the nerve.

The purpose of the study was to determine the expression of neurofilaments in the sciatic nerve, which regenerates after neurotomy and surgical suturing, and comparison of the effect of laser irradiation on different spectrum.

Material and methods. The experiment was conducted on 20 laboratory rats of the Wistar line (200–250 g), who were subjected to ether anesthesia in compliance with the rules of asepsis and antiseptics to a cross-section of the left sciatic nerve with end-to-end epineural suture suturing 30 minutes after neurotomy. The animals under study were divided into 4 groups of 5 each.

From the next day after the operation, the surgical area was irradiated for 10 days with a green spectrum laser with a wavelength of 560 nm (group 2) and 520 nm (group 3), as well as a blue spectrum laser with a wavelength of 480-450 nm (group 4). The laser irradiation (control) was not performed in the (group 1). The low-frequency LED laser "Spektr-LC", was used with exposure of 5 min. The study period was 90 days. Studies for somato-visceral sensitivity restoration were recorded by the animals' reaction to pain and temperature stimuli, and motor function using a moving tape.

During pathomorphological examination, the resulting nerve fragments were fixed in a 10 % solution of neutral buffered formalin (pH 7.4) for at least 24–36 hours, the material was proceeded according to the standard method and poured into paraffin. The serial histological sections with a thickness of 2–3 microns were made from paraffin blocks on a rotating microtome HM 325 (Thermo Shandon; England). The sections were stained with hematoxylin and eosin for review microscopy. In all cases, an immunohistochemical study (IGHD) was performed to determine the morpho-functional state of nerve cells and the features of regenerative abilities. Thereof, the sections were placed on Super Frost Plus adhesive glasses (Menzel; Germany). The citrate buffer with pH 6 and EDTA buffer pH 8, Vitro master Polymer Plus Detection System (Peroxidase), chromogen dab Quanto (Master Diagnostica; Spain) were used for high-temperature treatment of antigen epitopes. The mouse monoclonal antibodies to NEFL (Neurofilament Ab-1; clone 2f11; Thermo Fisher Scientific; USA) were used. The severity of the expression was assessed in accordance with the recommendations of D.J. Dabbs (2014) based on a visual-analog scale: 0 points – no color; 1 point (+) – weak color intensity; 2 points (++) – average color intensity; 3 points (+ + +) – high color intensity [6].

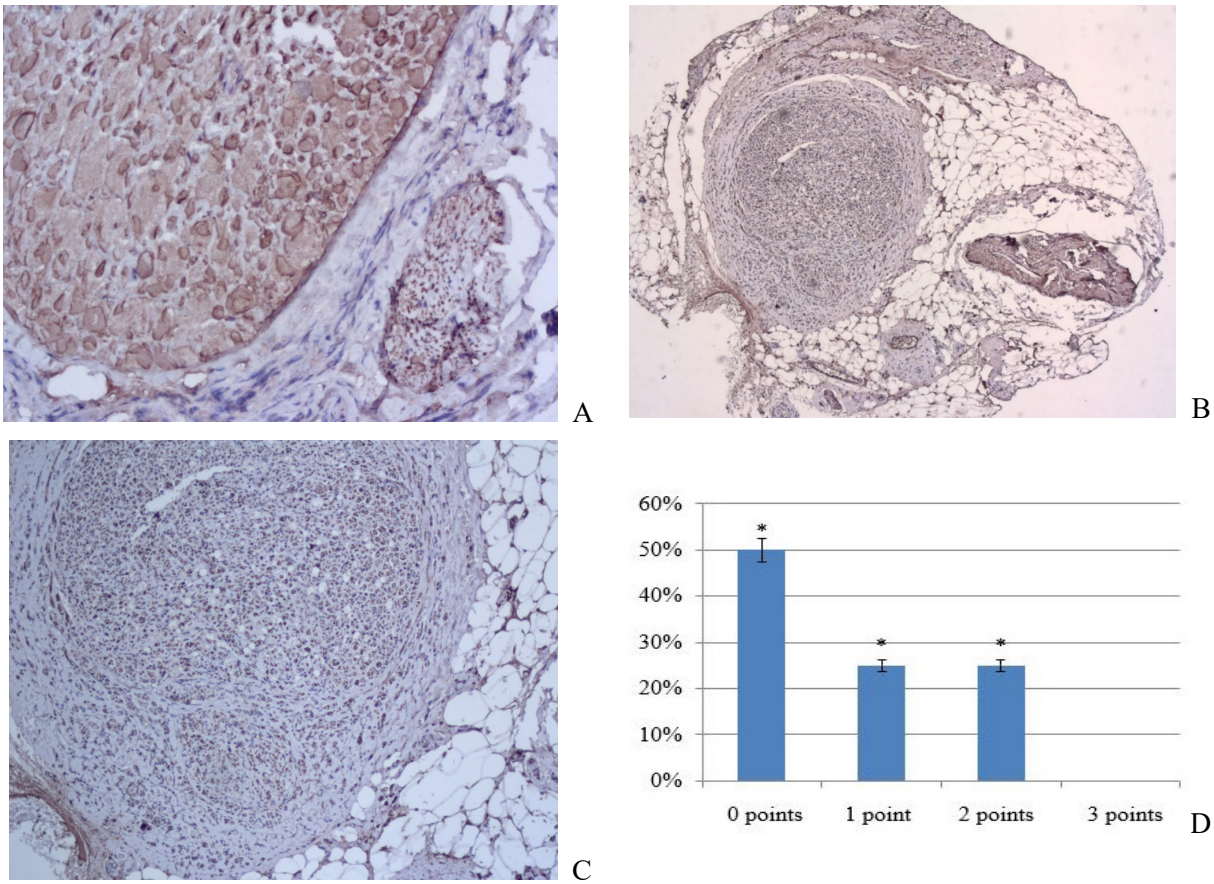
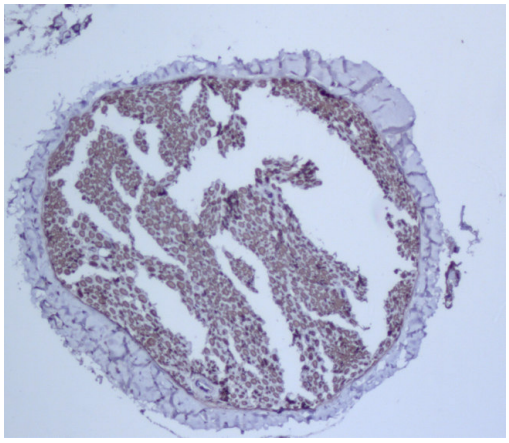
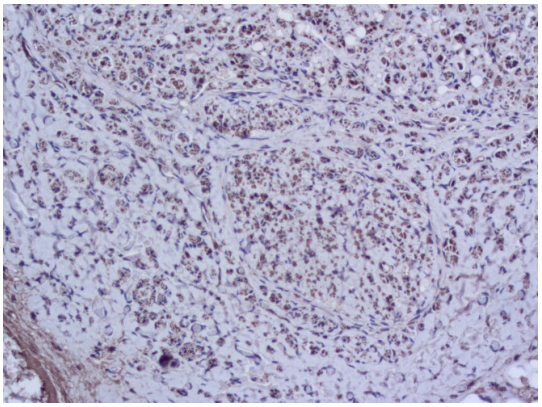


Fig. 1. Normal structure of the rat sciatic nerve. IGHD with monoclonal antibodies 2F11 to NEFL. Positive expression of NEFL in nerve cells. Magnification: A × 400. The rat sciatic nerve sutured 30 minutes after crosslinking; 90 days after crosslinking; laser correction (control) was not performed. IGHD with monoclonal antibodies 2F11 to NEFL. NEFL expression in nerve cells is weak and/or absent. Magnification: B ×50; C ×200. D – distribution (in %) of immunopositive cells by color intensity

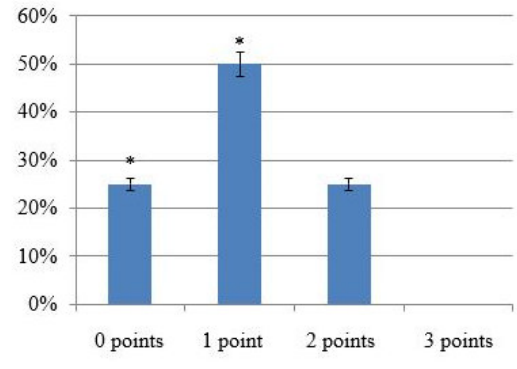
Results of the study and their discussion. Our studies have demonstrated that after crossing the sciatic nerve with its suturing, there were pronounced degenerative-dystrophic changes occurring in the nerve fibers, which are manifested by a decrease in the number of nerve fibers and their demyelination, spasm of perineural blood vessels, separation and swelling of the myelin sheath in the individual bundles of nerve fibers. There is no reaction to pain and temperature stimuli, and the animal spares the paw on the moving tape without stepping on it. Starting from day 15 the regenerative and reparative processes begin with a gradual restoration of blood supply to the nerve, remyelination of the nerve fibers. On day 30, the animals begin to react to temperature and pain stimuli and step on the operated paw on a moving tape. From day 60, there is a complete restoration of blood supply to the nerve, the return of the number of myelin nerve fibers, somato-visceral and motor sensitivity. The time of suturing the nerve after its intersection and application of the laser radiation was critical. Under the conditions of activity of the latter, a satisfactory recovery of the nerve occurred when it was stitched up to 30 minutes after crossing. An increase in the period of the nerve integrity surgical restoration led to a delay in the recovery period to 75–90 days. Based on these results, a comparison of the effect of radiation of different spectrum was made when the nerve was cross-linked exactly 30 minutes after intersection, and the recovery results were compared 90 days after the operation.



A

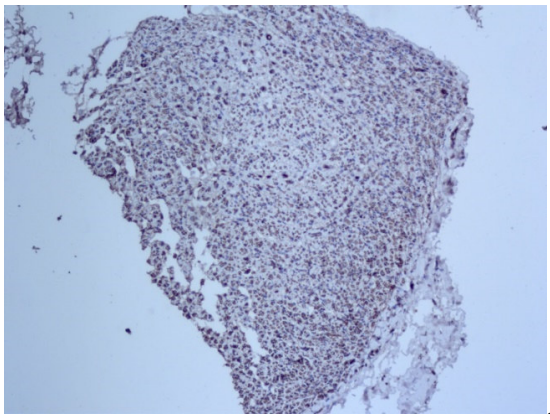


B

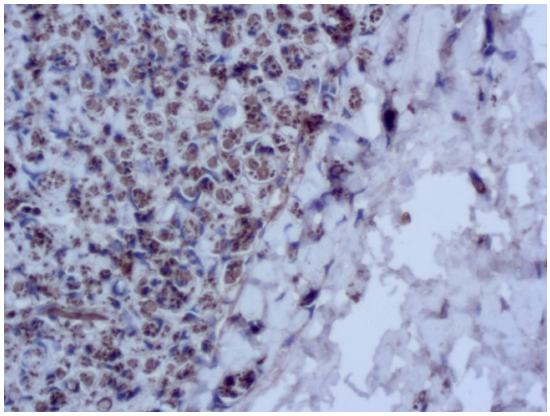


C

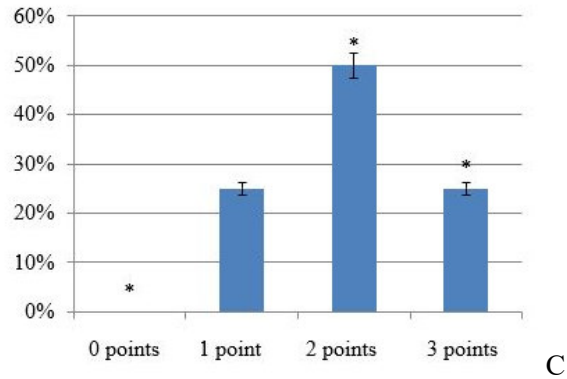
Fig. 2. The rat sciatic nerve sutured 30 minutes after crosslinking; 90 days after crosslinking; laser correction of the green spectrum with a wavelength of 560 nm. IGHD with monoclonal antibodies 2F11 to NEFL. NEFL expression in nerve cells is weak and/or absent. Magnification: A ×50; B ×200. C – distribution (in %) of immunopositive cells by color intensity; * – $p<0.05$ compared to the control.



A



B



C

Fig. 3. The rat sciatic nerve sutured 30 minutes after crosslinking; 90 days after crosslinking; laser correction of the green spectrum with a wavelength of 520 nm. IGHD with monoclonal antibodies 2F11 to NEFL. NEFL expression in nerve cells of varying severity. Magnification: A ×50; B ×400 C – distribution (in %) of immunopositive cells by color intensity; * – $p<0.05$ compared to the control.

Figure 1 demonstrates a normal structure of the rat sciatic nerve. NEFL expression was reflected on the entire section of nerve fibers in axons, the color intensity was 3 points and rat sciatic nerve, which was sutured 30 minutes after crossing, on the 90th day of follow-up without laser irradiation. The main type of the regenerated nerve fiber was myelinated fiber of small diameter (2–3 microns), the number and density of axon myelin sheath were low, and connective tissue overgrowth was high. NEFL expression is expressed weakly (up to 25 % of neurons) or absent, and the staining intensity of immunopositive cells is 1–2 points.

Fig. 2 demonstrates the structure of the rat sciatic nerve, which was sutured 30 minutes after crossing, on the 90th day of observation with laser correction of the green spectrum with a wavelength of 560 nm. NEFL expression in most cells is weakly expressed – up to 50 % of cells have a color intensity of 1 point. A small proportion (up to 25 %) of cells have an average color intensity (2 points); the rest do not have it.

Fig. 3 demonstrates the structure of the rat sciatic nerve, which was sutured 30 minutes after crossing, on the 90th day of observation with laser correction of the green spectrum with a wavelength of 520 nm. NEFL expression was more reflected than in the previous groups, most cells had a staining intensity of 2 points (up to 50 %), and some cells (large ganglion cells with high staining intensity) possessed 3 points (up to 25 %). The remaining cells were 1–2 points of intensity.

Fig. 4 demonstrates the structure of the rat sciatic nerve, which was sutured 30 minutes after crossing, on the 90th day of observation with laser correction of the blue spectrum with a wavelength of 450–480 nm. In this group, NEFL expression was expressed to the maximum extent, and the historachitectonics of the sciatic nerve almost did not differ from the normal structure. More than 75 % of the cells were 3 points of color intensity, the rest – 2 points. The majority (up to 75 %) were of high staining Intensity (3 points) among large ganglion cells.

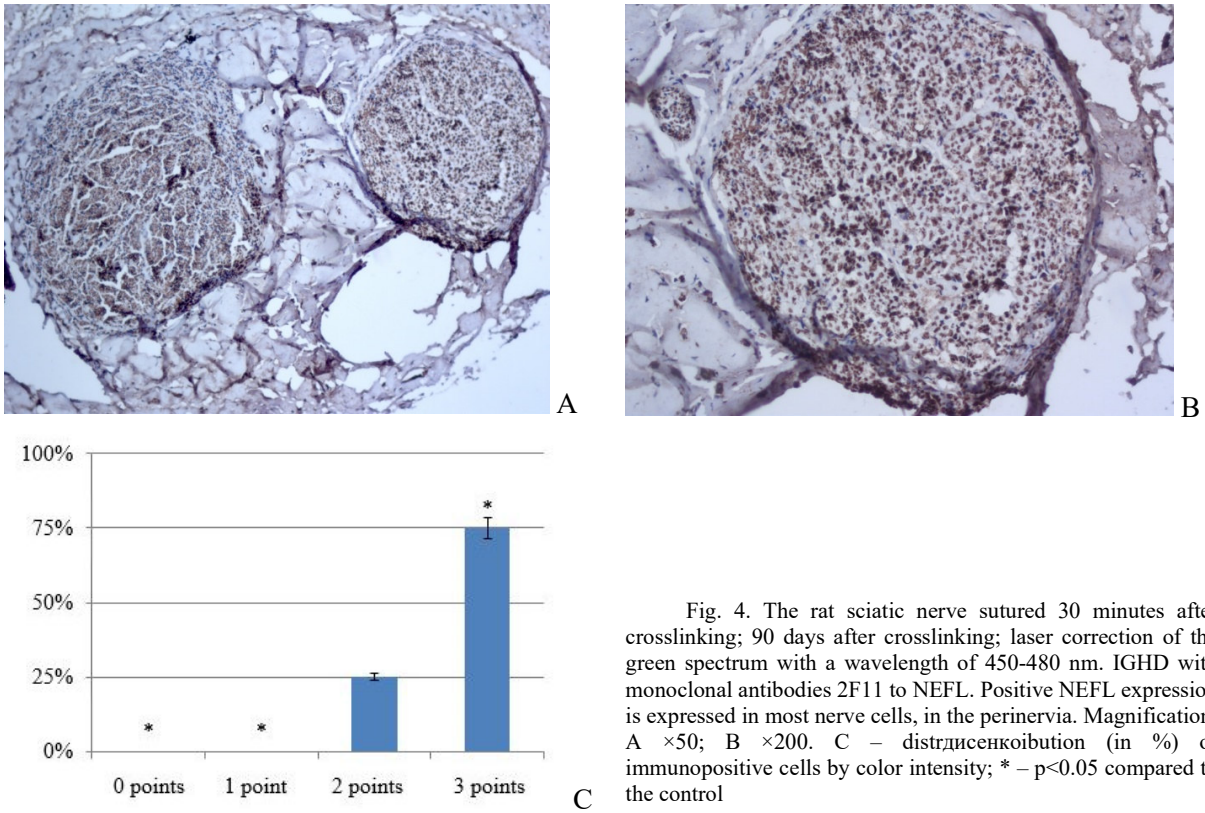


Fig. 4. The rat sciatic nerve sutured 30 minutes after crosslinking; 90 days after crosslinking; laser correction of the green spectrum with a wavelength of 450-480 nm. IGHD with monoclonal antibodies 2F11 to NEFL. Positive NEFL expression is expressed in most nerve cells, in the perinervia. Magnification: A ×50; B ×200. C – distгдисенкоibution (in %) of immunopositive cells by color intensity; * – $p<0.05$ compared to the control

Thus, it was shown that the intensity of immunopositive staining on NEFL correlates well with the dynamics of restoration of sciatic nerve structure and function, which is consistent with data from other studies [5, 9]. The staining intensity was lowest in group 1 (control), where laser irradiation was not used. The morphological picture corresponded to that described in other studies, namely, signs of demyelination were preserved, the regenerated fibers had a small diameter and a certain density, but active fibrosis was determined [4].

Likewise, our study supports the opinion of M. Hendry et al. (2019) regarding the high informative value and ability of neurofilament staining by IGHD, which correlates with electron microscopy data, which is particularly useful for counting unmyelinated axons in the restored peripheral nerve [7]. Regarding

the cause of the impaired neurofilament formation in nerve damage, the attention should be paid to importance of the epigenetic modifications, such as DNA methylation, which can change gene expression in response to environmental factors [3]. A total of 38 sites in neurofilament genes, amyloid precursor protein beta (APP), microtubule-associated tau protein (MAPT), were methylated when nerve tissue was damaged. The process of DNA methylation reduces the expression of the corresponding mRNAs and may have consequences for development of the neurodegenerative disorders after traumatic damage to the nervous tissue.

According to M. M. Mandelbaum-Livnat et al. (2016), there is a considerable interest in the potential therapeutic value of laser phototherapy (photobiomodulation) to enhance the regeneration of severely damaged peripheral nerves [8, 11]. The laser phototherapy has a rapid protective effect not only on nerve regeneration, but also in the early stages of muscle atrophy can preserve denervated muscle by maintaining creatine kinase activity and the number of acetylcholine receptors (AChR). Under the conditions of laser phototherapy, as early as forty-five days after sciatic nerve damage, all rats regained normal walking, which was directly related to the duration of radiation sessions (from 3 to 7 minutes). The laser irradiation was accompanied by a large number of neuronal fibers that were thicker than 4 microns.

Together with such studies, our results convincingly demonstrated a positive effect of laser radiation, better nerve regeneration, and active growth of neurofilaments. In addition, our study revealed the greatest positive effect from the use of laser irradiation of the blue spectrum with a wavelength of 450–480 nm.

Conclusion

1. Application of laser irradiation in the event of sciatic nerve regeneration and surgical suturing increases sufficiently the neurofilament expression (NEFL) based on the results of the immunohistochemical study.

2. Application of the laser irradiation of blue spectrum with a wavelength of 450-480 nm was the most effective, which has allowed to renew morpho-functional features of the crossed sciatic nerve in full.

References

1. Svyrydiuk RV, Shepitko VI, Shepitko KV, Klypachenko IV. Kharakterystyka strukturnykh komponentiv stovbura sidnychnoho nerva pry vvedenni kriokonservovanoi platsenty u shchuriv. Svit medytsyny ta biolohii. 2018; № 4: 202–207. doi: 10.26724/2079-8334-2018-4-66-202-207. [in Ukrainian].

2. Yashchyshyn ZM, Zaiats LM, Nebesna ZM, Poliuk MI, Rohuzhynska VH, Kerniakevych SIa. Morfofunktsionalni zminy v strukturi sidnychnoho nerva pislia neirotomii z ushyvanniam epinevralnym shvom cherez rizni promizhky chasu ta podalshoiu lazernoiu korektsiiu. Aktualni problemy transportnoi medytsyny. 2020. № 2 (60):85–91 [in Ukrainian]. Doi http://dx.doi.org/10.5281/zenedo.3967612.

3. Abu Hamdeh S, Ciuculete DM, Sarkisyan D, Bakalkin G, Ingelsson M, Schiöth HB, Marklund N. Differential DNA Methylation of the Genes for Amyloid Precursor Protein, Tau, and Neurofilaments in Human Traumatic Brain Injury. J Neurotrauma. 2021 Jun 15;38(12):1679–1688. doi: 10.1089/neu.2020.7283.

4. Chen J, Yang R, Li H, Lao J. Green Tea Polyphenols Promote Functional Recovery from Peripheral Nerve Injury in Rats. Med Sci Monit. 2020 Aug 27; 26: e923806. doi: 10.12659/MSM.923806.

Dang Do AN, Sinaii N, Masvekar RR, Baker EH, Thurm AE, Soldatos AG, Bianconi SE, Bielekova B, 5. Porter FD. Neurofilament light chain levels correlate with clinical measures in CLN3 disease. Genet Med. 2021 Apr;23(4):751–757. doi: 10.1038/s41436-020-01035-3. Epub 2020 Nov 26. PMID: 33239751; PMCID: PMC8035240.

6. Dubois M, Lalonde R, Julien JP, Strazielle C. Mice with the deleted neurofilament of low-molecular-weight (Nefl) gene: 1. Effects on regional brain metabolism. J Neurosci Res. 2005 Jun 15;80(6):741–50. doi: 10.1002/jnr.20449. PMID: 15742362.

7. Hendry JM, Alvarez-Veronesi MC, Chiang C, Gordon T, Borschel GH. Neurofilament-histomorphometry comparison in the evaluation of unmyelinated axon regeneration following peripheral nerve injury: An alternative to electron microscopy. J Neurosci Methods. 2019 May 15; 320:37–43. doi: 10.1016/j.jneumeth.2019.03.006.

8. Mandelbaum-Livnat MM, Almog M, Nissan M, Loeb E, Shapira Y, Rochkind S. Photobiomodulation Triple Treatment in Peripheral Nerve Injury: Nerve and Muscle Response. Photomed Laser Surg. 2016 Dec;34(12):638–645. doi: 10.1089/pho.2016.4095. PMID: 28001757.

9. Sainio MT, Ylikallio E, Mäenpää L, Lahtela J, Mattila P, Auranen M, Palmio J, Tyynismaa H. Absence of NEFL in patient-specific neurons in early-onset Charcot-Marie-Tooth neuropathy. Neurol Genet. 2018 Jun 5;4(3): e244. doi: 10.1212/NXG.0000000000000244. PMID: 29888333; PMCID: PMC5991776.

10. Sta M, Cappaert NL, Ramekers D, Baas F, Wadman WJ. The functional and morphological characteristics of sciatic nerve degeneration and regeneration after crush injury in rats. J Neurosci Methods. 2014 Jan 30; 222:189–98. doi: 10.1016/j.jneumeth.2013.11.012. Epub 2013 Nov 25. PMID: 24286698.

11. Svyrydiuk RV, Shepitko KV, Shepitko VI, Raskaley TYa. Morphometric characteristics of the sciatic nerve trunk in the acute aseptic inflammation in rats. World of Medicine and Biology. 2018; № 2(64):173–175. doi: 10.26724 / 2079-8334-2018-2-64-173-175.

Стаття надійшла 30.07.2021 р.