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Effect of Long-term Peripheral Nerve Stimulation on Neuro-Muscular Complex Morphologic Recovery in Experiment

Efecto de la Estimulación del Nervio Periférico a Largo Plazo sobre la Recuperación Morfológica del Complejo Neuromuscular en Experimento

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SUMMARY: Peripheral nerve injury is an extremely important medical and socio-economic problem. It is far from a solution, despite on rapid development of technologies. To study the effect of long-term electrical stimulation of peripheral nerves, we used a domestically produced electrical stimulation system, which is approved for clinical use. The study was performed on 28 rabbits. Control of regeneration was carried out after 3 month with morphologic techniques. The use of long-term electrostimulation technology leads to an improvement in the results of the recovery of the nerve trunk after an injury, both directly at the site of damage, when stimulation begins in the early period, and indirectly, after the nerve fibers reach the effector muscle.

KEY WORDS: Experiment; Peripheral nerve injury; Neuro-muscular complex; Long-term electrostimulation; Morphology.

INTRODUCTION

Traumatic injuries of peripheral nerves (PN) occur with a frequency of 13 to 23 per 100,000 per year in the general population (Robinson *et al*., 2022). In the overall pattern of traumatic injuries, 5 % of patients have peripheral nerve injuries and 1 % have brachial plexus injuries (Padovano *et al*., 2022). Peripheral nerve damage is accompanied by impaired movement and sensitivity, neuropathic pain syndromes. Despite the possibilities of early diagnosis and the modern development of microsurgical techniques, complete recovery of peripheral nerves after injuries never occurs (Miclescu *et al*., 2019; Bellaire *et al*., 2021). The problem of restoring not only the structure, but also the function of peripheral nerves remains relevant not only in the medical, but also in the socio-economic plan (Aman *et al*., 2022).

Peripheral nerve recovery is influenced by a variety of factors, such as the type and level of damage, the condition of surrounding tissues, the timing of surgery, changes in spinal cord motoneurons and effector organs (Gordon, 2021).

After transection of the nerve, axons that lose contact with the neuron body undergo Wallerian degeneration. Even after performing a microsurgical suture of the nerve, the phenomena of degeneration continue and axons have to sprout along the entire distal end of the damaged nerve, in fact along the connective tissue matrix. After the end of degenerative processes, regeneration processes begin. Schwann cells form fibers in the direction from the proximal end of the nerve to the distal end, sprouting the area of the nerve damage up to the target organ (Liu *et al*., 2019, Nocera & Jacob, 2020).

The time during which the sprouting of nerve fibers takes place is of critical importance, since denervation of the muscle, even after immediate restoration of the integrity

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of the nerve, leads to the loss of 65 % of the functional potential of the muscle. In the case of chronic denervation of the muscle, the loss of functional potential decreases to 10 %. Another factor is the number of Schwann cells and their ability to migrate from the proximal end of the damaged nerve to the distal end and participate in the reinnervation of the muscle (Wang *et al*., 2019; Aidharma *et al*., 2022).

In addition, the deficiency of motor units may arise due to insufficient number of axons due to the peculiarities of reconstructive surgeryon the nerve trunk (for example, with nerve transfer, autoplasty, size differences and histoarchitectonics of the nerve endings) (Rayner *et al*., 2020).

The effect of the electromagnetic field on nerve tissue is related to the transmembrane potential. During influence of electromagnetic field, neuron changes its resting membrane potential to an action potential. The cell membrane is depolarized or hyperpolarized, which causes excitation or inhibition of electrical impulse conduction (Fu *et al*., 2020; Jin *et al*., 2022).

Axon regeneration through the nerve suture line is slow and axons sprout through it within 3-4 weeks. Regeneration is accelerated by electrical stimulation with a frequency of 20 Hz within 1 hour after performing a nerve suture, and stimulation of regeneration occurs mainly due to an increase in the rate of axon sprouting and not their number (Javeed *et al*., 2021).

The positive effect of transcutaneous electromyostimulation and temporary intraoperative neurostimulation is known (Shapira & Midha, 2015; Zuo *et al*., 2020). The question of the effect of long-term invasive electric neurostimulation on the recovery of the neuromuscular complex as a functional unit in case of peripheral nerve injuries remains open.

MATERIAL AND METHOD

This study was conducted in strict accordance with the recommendations of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The research protocol with animal experimentation was approved by the Scientific Ethics Committee of State Institution "Romodanov Neurosurery Institute Of NAMS Of Ukraine" (Protocol Number: 17/2022). All surgery was performed under general anesthesia and every effort was made to minimize suffering.

The study was conducted on 28 rabbits of the vivarium of the State Institution "Romodanov Institute of Neurosurgery NAMS of Ukraine". Operative interventions was performed in aseptic conditions and in compliance with the rules of bioethics.

Under general anesthesia with solutions of ketamine hydrochloride 10 % (50 mg/kg), xylazine hydrochloride 2 % (5 mg/kg) and atropine sulfate (0.05 mg/kg), surgical approach to the sciatic nerve in a rabbit was performed.

Distribution of groups depending on the method of surgical intervention:

Group 1 (n=7), control: suture of the sciatic nerve and implantation of the non-working antenna of the electrical stimulation device. This group of animals will not be stimulated.

Group 2 (n=7): sciatic nerve suture + implantation of an electric stimulator antenna and the beginning of stimulation on the 2nd day.

Group 3 (n=7): sciatic nerve suture + implantation of an electric stimulator antenna and the beginning of stimulation at a time point that will coincide with the beginning of signs of reinnervation of the effector muscles.

Group 4 (n=7): autograft of the sciatic nerve + implantation of an electric stimulator antenna and the beginning of stimulation at a time point that will coincide with the beginning of signs of reinnervation of the effector muscle.
Surgical procedure. In group 1, we perform approach to SN in aseptic conditions, at a distance of 30±1.5 mm from the point of its exit from the pelvic cavity, cut the nerve with blade and make epineural suture (monofilament polyamide, 9.0). Antenna of electrostimulation device (NeySi-3M) (Fig. 1) was implanted subcutaneously (at thigh level) and electrodes was sutured to epineurium by atraumatic sutures (monofilament polyamide, 9/0) (Fig. 1B). Layer-by-layer suturing of the wound. The same procedure was performed in group 2 and group 3. In group 4, nerve gap with a length of up to 10±2 mm was cut out with the help of a blade (at a distance of 30±1.5 mm from the point of its exit from the pelvic cavity). After turning the fragment by 180º, it was again sewn between the ends of the SN. 3–6 epineural sutures were applied using an atraumatic needle with a 9/0 monofilament polyamide using an operating microscope (×12 magnification). This method simulates the nerve autografting operation in the clinic.

The NeySi-3M neurostimulation system is a Ukrainian-made neurostimulator that is partially implantable, is used in neurosurgery and is intended for long-term electrical stimulation of peripheral nerves and plexuses, the

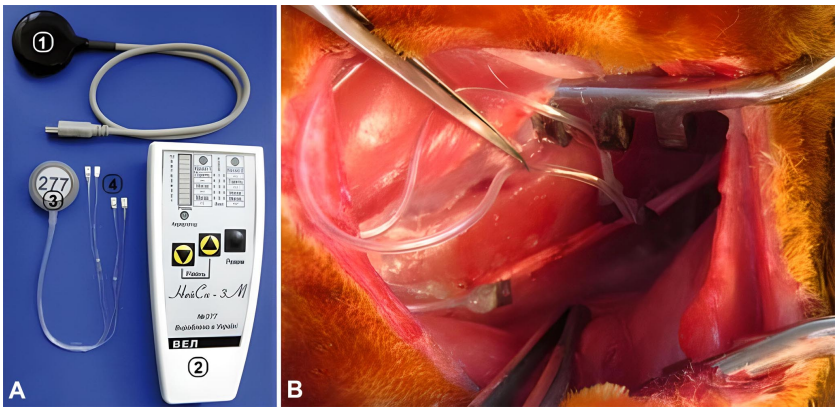

Fig. 1. NeySi-3M neurostimulator. A - general view. Nonimplantable part: 1. transmission antenna, 2. pulse generator unit (combined with the control panel); Implantable part: 3. receiving antenna, 4. electrodes. B - intraoperative photo: electrodes was fixed to the epineurium of sutured sciatic nerve.

spinal cord, and areas of the brain, with the aim of relieving neuropathic pain, as well as restoring the functions of damaged structures of the central and peripheral nervous systems (nerves, plexuses, brain and spinal cord).

The NeSi-3M electrostimulation device is included in the State Register of Medical Equipment and Medical Products of Ukraine and isapproved for use in medical practice. State registration certificate No. 7439/2008.

Electrostimulation protocol. During the electrical stimulation session, voltage pulses from the device's generator were transmitted through the transmitting antenna, which was applied to the surface of the skin above the receiving antenna implanted in the animal's body. From the receiving antenna, voltage pulses were sent through a thin cable to the electrodes, which are sewn to the epineurium of the sciatic nerve. The electrostimulation mode was used with a variable frequency of pulses per cycle: half of the T period – pulse generation, half of the T period – pulse pulse in the function, T – from 0.5 s to 15 s, minimum frequency (F min.) – 2 Hz, maximum frequency (F max.) – 120 Hz, and with a fixed frequency of 20 Hz and 80 Hz. The amplitude of the pulses in all modes with a load resistance of 10 kΩ was from 8 V to 20 V. Electrical stimulation sessions lasting 5 minutes per day were conducted every day.

Control of regeneration was carried out after 8 weeks. After the animal was anesthetized, access to the sciatic nerve was performed, and it was removed together with the calf muscles. After the collection of materials, tissues are removed from the experiment by injection of a lethal dose of drugs for anesthesia.

The material was fixed in a 10 % solution of buffered formalin. Longitudinal frozen sections were made on a MK-2 microtome-cryostat, after which impregnation with silver nitrate was carried out by the Bylshovsky method in modication of Chaikovsky (Chaikovsky *et al*., 2016).

Muscle fragments were processed using standard paraffin-embedded techniques. Paraffin sections of 5-7 mm were made on a rotary microtome Sakura Accu-cut SRM 200 (Sakura, Japan), stained with hematoxylin-eosin. The obtained micro samples were photographed on an Olympus BX51 microscope (Olympus, Japan), and processed with the ImageJ V1.50 biomedical image processing program (NIH, USA).

The values of the indicators were calculated using the methods of descriptive statistics. The normality of data distribution was checked by the Shapiro-Wilk W-test. Since the test did not confirm that the distribution law was normal, for multiple intergroup comparisons of the mean values of independent groups, the non-parametric Kruskal-Wallis H-test was used, followed by pairwise comparisons of groups using the U-Mann-Whitney test (Mann– Whitney U-test. The average values were presented as M (25 %; 75 %) (n), where M is the median; (.) is the interquartile range. The electronic database was created using the MS Excel 2013 program. Statistical analysis and graphical presentation of results was performed using the Prizm v. 8.0 (GraphPad, USA, free trial) and STATISTICA 7 (StatSoft Inc. (2003)) software package.

RESULTS

Three month after the injury, signs of regeneration was macroscopically present in the area of surgical intervention. Histological examination revealed that the suture site included numerous newly formed nerve fibers, blood vessels, and an implanted stimulator antenna. Newly formed nerve fibers (Fig. 2A) went from the proximal (central segment) through the injury site and grew into the distal (peripheral segment). Newly formed nerve fibers are located relatively evenly, but in a disorderly manner. A large number of thin nerve fibers deviate from the longitudinal axis of the nerve and are located at large angles. The number of blood vessels is insignificant. In the suture site, there are no signs of fibrosis or excessive growth of connective tissue around

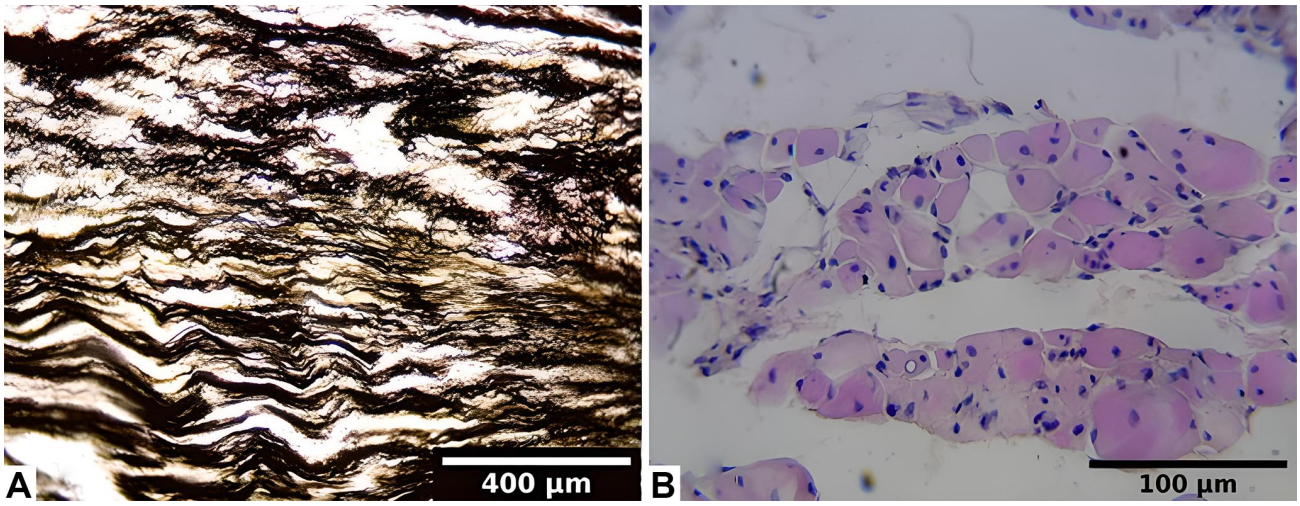

Fig. 2. A . Suture site in animals of the I group. Chaotic, relatively uniform arrangement of a large number of nerve fibers. Impregnation with silver nitrate. B. Fragment of the calf muscle of animals of the I group. Muscle fibers of medium and small diameter. Hematoxylin-eosin staining.

the antenna. In the area of suture site, the number of newly formed nerve fibers was 8082,1 (7696,7; 8687,6)/µm^2.

During the histological examination of calf muscles in animals of this group, muscle fibers of medium and small diameter forming bundles were found (Fig. 2B). Most of the muscle fibers have eosinophilic cytoplasm and peripherally located nuclei, but there is a moderate number of luminal fibers and fibers of small diameter with their centrally located nuclei. The cross-sectional area of the muscle fibers was 295.3 (180,7-415,7) /µm^2 (Median; 95 % CI of Median).

Similarly, to the previous group, in the area of injury, the animals of this II group had a suture site consisting of thin newly formed nerve fibers that sprouted from the injury site and grew to the distal segment of the injured nerve, a small number of blood vessels, and an implanted antenna of the stimulator. The number of newly formed fibers is significant. The unevenness of the nerve fibers of the suture site area is noted: a significant number of nerve fibers pass near and along the antenna of the stimulator (Fig. 3). Such nerve fibers are oriented along the antenna and are placed in an orderly manner. At a certain distance from the stimulator, nerve fibers have a tortuous course, are arranged chaotically, deviate from the longitudinal axis of the nerve. The number of newly formed nerve fibers was 11705 (11302,2; 12513,1) 1/µm^2, which was significantly ($p<0.0004$) more than the similar indicator in animals of the 1st group (Fig. 3A).

High density of newly formed nerve fibers around the area of implantation of the stimulating electrode. In the area of the suture site bordering the peripheral segment of the nerve, there is a large number of newly formed nerve fibers, which were placed unevenly, with a high density in the central part of the nerve trunk, which corresponds to the depth of implantation of the stimulator (Fig. 3B).

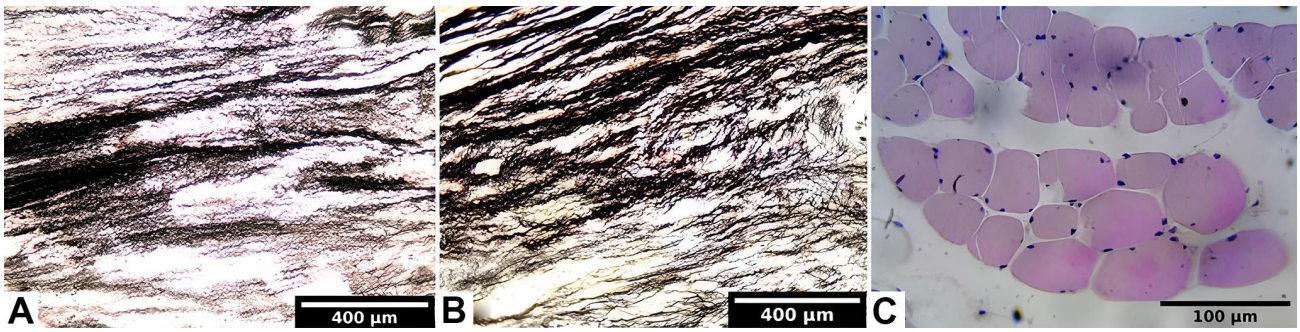

Fig. 3. A. Suture site of II group animals. B. Suture site and peripheral segment of the sciatic nerve of animals of the II group. A large number of newly formed nerve fibers. Impregnation with silver nitrate. C. Fragment of the calf muscle of animals of the II group. Large diameter muscle fibers. Hematoxylin-eosin staining.

During the histological examination of the calf muscle, bundles of muscle fibers with a predominance of muscle fibers of large and medium diameter with eosinophilic cytoplasm and peripherally located nuclei were noted in their composition (Fig. 3C). The cross-sectional area of the muscle fibers was 1094,9 (791,3; 1408,4) 1/μm^2, which was reliable ($p<0.0001$) for a similar indicator of animals from the 1st group.

According to the results of the research, the animals of III group had numerous thin and thick newly formed nerve fibers in the area of the suture site, which went from the central segment and grew into the peripheral segment of the injured nerve trunk. Nerve fibers were located relatively unevenly, relatively disorderly (Figs. 4A,B). A significant number of fibers in the peripheral part of the neuroma retained signs of longitudinal orientation; on the other hand, in the middle part of the suture site, a large number of thin nerve fibers had a chaotic arrangement, deviated at significant angles from the longitudinal axis of the nerve. The specific number of newly formed nerves in the area of the suture site was 9788 (9283; 10394,0) 1/μm^2, which was insignificantly ($p=0.38$) more than the similar indicator in animals of the 1st group and insignificantly ($p=0.39$) less than this indicator in animals of the II group.

Histological examination of the calf muscle revealed a polymorphism of the morphological pattern: there are medium-diameter fibers with eosinophilic cytoplasm and eccentrically located nuclei, as well as small-diameter fibers with centrally located nuclei and small fibers with many nuclei (Fig. 4C). The cross-sectional area of the muscle fibers was 570.1 (343.2; 761.2) 1/μm^2, which was significantly ($p<0.0001$) higher than the similar indicator in animals of the I group, but was significantly lower ($p<0.0001$) than the same indicator in animals of the II group.

According to the results of histological examination of the injured nerve trunk in group IV, it was established that proximal and distal to suture site were formed in the area of injury in the animals (between the central segment of the nerve and the insertion area and between the insertion area and the peripheral segment of the nerve, respectively). In the area, proximal to suture site, there were thick and thin newly formed nerve fibers that germinated at the site of the injury and grew into the area of the insertion. An uneven arrangement of nerve fibers in the composition of the suture site is noted. Most of the fibers are placed in a relatively orderly manner, maintaining a longitudinal orientation (Fig. 5A). The specific number of fibers was 9223,5 (8880.3; 9586,7) 1/μm^2, which was significantly ($p=1,0$) more than the corresponding indicator in animals of the I group, but this indicator was significantly less ($p=0,043$) for a similar indicator in animals of the II group and did not differ significantly ($p=1.0$) from a similar indicator in animals of the III group.

In the area of the distal suture site (Fig. 5B), the presence of a small number of thick and a significant number of thin nerve fibers growing into the peripheral segment of the nerve trunk was noted. The placement of fibers in the area of the insertion is relatively uniform, however, not all fibers reach the peripheral segment of the nerve trunk, which causes its uneven reinnervation. The average number of thin newly formed nerve fibers deviates from the longitudinal axis of the nerve, has a transverse or recurrent course. The number of fibers was 6559,3 (6357,5; 6962,9) 1/μm^2, which was significantly less than the similar indicator both in the proximal suture site in animals of this group ($p<0.012$) and in animals II ($p<0.0001$) and III ($p=0.0005$)) experimental groups and did not differ significantly ($p=0.49$) from the similar indicator in animals of the 1st group.

During the histological examination of the calf muscle on the side of the injury, single fibers of medium diameter and numerous small fibers that were part of the bundles were found (Fig. 5C). Numerous groups of adipocytes were also found in the spaces between muscle fibers. The cross-sectional area of muscle fibers was 355.1 (253.3-492.1) 1/μm^2, which was not significantly different

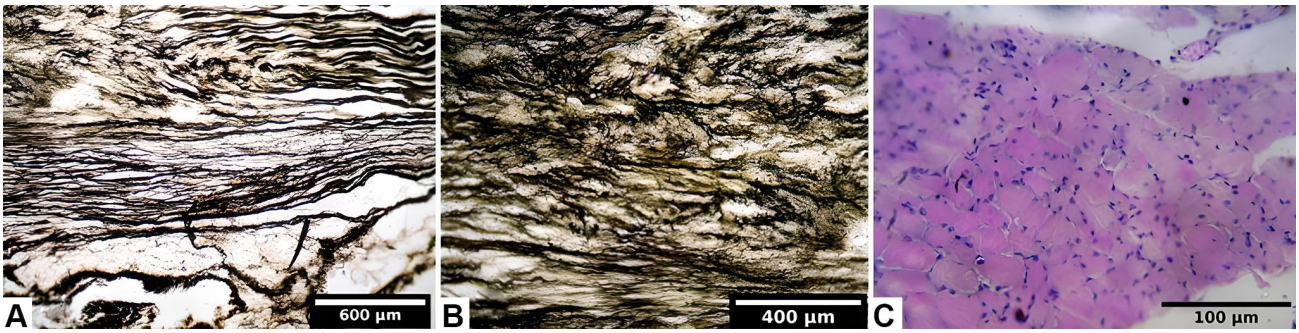

Fig. 4. A, B. Suture site of animals of the III group. Chaotic, uneven arrangement of nerve fibers. Impregnation with silver nitrate. C - Fragment of the calf muscle of animals of the III group. Muscle fibers of medium diameter. Hematoxylin-eosin staining.

from the similar indicator in animals of group I (p=1.0) and group III (p=0.35) but this indicator was significantly lower than the similar indicator in animals of II experimental group (p<0.0001).

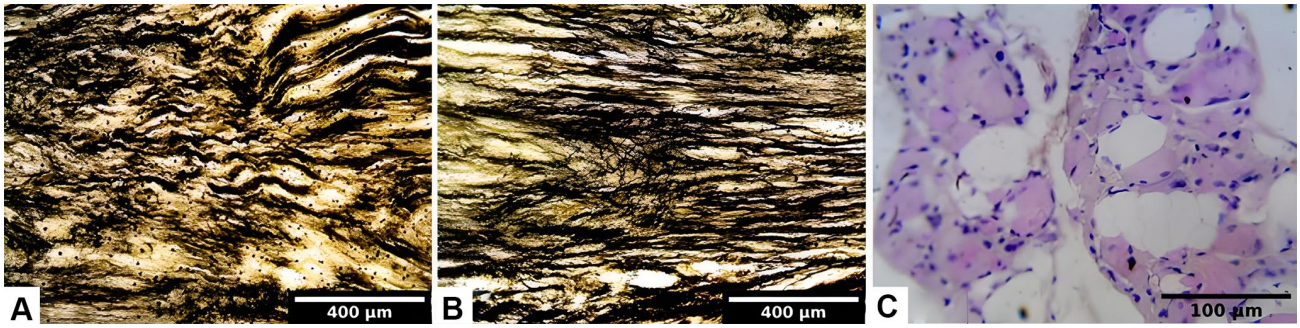

Fig. 5. A. Proximal suture site of IV group animals. Chaotic, uneven arrangement of nerve fibers. Impregnation with silver nitrate. B. Distal suture site of IV group animals. Chaotic, uneven arrangement of nerve fibers. Impregnation with silver nitrate. C. Fragment of calf muscle of IV group animals. Muscle fibers of medium and small diameter, atrophied fibers. Hematoxylin-eosin staining.

DISCUSSION

Histological research data indicate the presence of reactive changes in response to damage to the nerve trunk both in the area of direct injury and in the effector organ along with the location of neuron bodies, which is described in the classical literature. It is also common knowledge that suture site form in areas of severe damage to nerve trunks, especially after damage in the form of a complete section. The nature of the changes that were revealed during the study of fragments of the nerve trunk and muscle of animals of the I group are typical and corresponds to the picture of the long-term consequences of nerve injury grade V (according to Sunderland), neurotmesis (according to Seddon) after nerve suture, which is well described in the literature sources. Such changes include the relatively disordered arrangement of newly formed nerve fibers in the area of nerve injury with the sprouting of nerve fibers into the peripheral segment, restoration of the shape of neurons, incomplete restoration of their size, the appearance of chromatophilic substance of neurons of the spinal cord, the central location of the nuclei of neurons and the average diameter of muscle fibers. When examining the material of the animals of the II experimental group, changes similar to those of the I group were also found. However, the animals of this group had a greater number of nerve fibers in the area of the injury, at the same time with a greater degree of their orderliness, especially around the area of implantation of the stimulating electrode, which, in combination with the presence of full-fledged muscle fibers of large diameter indicates a more successful regeneration in animals of this group. This effect can be explained both due to the positive effect of the electric field of the implanted electrode in the area of the injury in the form of stimulation of the sprouting of a larger number of ordered nerve fibers, and due to the indirect stimulating effect on successfully reinnervated muscle fibers.

It was also established that there was no irritating or damaging effect of stimulation both in the place of electrode implantation which, however, requires further research. In the animals of the III experimental group, in which stimulation with an implanted electrode was carried out in the remote period after the injury, histological examination of the material of various organs also showed a typical pattern of late changes both at the site of the injury and in distant organs. However, the degree of severity of these changes differed from that in animals of the II group: the degree of orderliness and orientation of the nerve fibers approached those of the animals of the I group. However, a higher index of the cross-sectional area of muscle fibers may probably indicate a mediated proregenerative effect not on the sprouting of nerve fibers through the injury site, but on muscle recovery, since the reaching of muscle fibers by nerve fibers and the beginning of electrical stimulation coincide in times. Small differences in the number of nerve fibers between the III and I groups of animals can be explained by the late start of stimulation, which does not coincide in time with the moment of sprouting of nerve fibers through the injury site, since this process occurs earlier. In animals of group IV, where autoplasty was performed with the beginning of stimulation in the remote period after the injury, morphological changes and morphometric parameters in the proximal suture site are similar to those in animals of group III, which can also be explained by the discrepancy in the time of fiber sprouting and the beginning of stimulation. A morphological study of the muscle revealed changes characteristic of incomplete, uneven restoration of its structure in the form of the appearance of groups of small fibers, an increase in the number of adipocytes, which occurs due to the delay and unevenness of the reinnervation of the muscle, although the presence of muscle fibers of medium diameter, forgiving

cross section which does not differ from the control may also indicate a pro-regenerative mediated effect of late electrical stimulation on the recovery of those areas of the muscle, the reinnervation of which was successful.

CONCLUSIONS

Thus, the application of electrical stimulation of an injured peripheral nerve with the implantation of a stimulator antenna directly at the site of injury does not have a negative damaging effect on the surrounding tissues. The use of this technology leads to an improvement in the results of the recovery of the nerve trunk after an injury, both directly at the site of damage, when stimulation begins in the early period, and indirectly, after the nerve fibers reach the effector muscle. For a more in-depth study of the effects of electrical stimulation on the regeneration of the neuromuscular complex, it is necessary to study changes in nerves, muscles, and the spinal cord at the subcellular level.

PETRIV, T; ALMHAIRAT, R. M. D.; LIKHODIEVSKIY, V.; LUZAN, B.; TSYMBALIUK, Y.; VASLOVYCH, V.; MALYSHEVA, T. & TSYMBALIUK, V. Efecto de la estimulación del nervio periférico a largo plazo sobre la recuperación morfológica del complejo neuromuscular en experimento. *Int. J. Morphol., 42(1)*:166-172, 2024.

RESUMEN: La lesión de los nervios periféricos es un problema médico y socioeconómico extremadamente importante. Sin embargo, y a pesar del rápido desarrollo de las tecnologías, aún no tiene solución. Para estudiar el efecto de la estimulación eléctrica a largo plazo de los nervios periféricos, utilizamos un sistema de estimulación eléctrica de producción nacional, que está aprobado para uso clínico. El estudio se realizó en 28 conejos. El control de la regeneración se realizó a los 3 meses con técnicas morfológicas. El uso de tecnología de electro estimulación a largo plazo conduce a una mejora en los resultados de la recuperación del tronco nervioso después de una lesión, tanto directamente en el lugar del daño, cuando la estimulación comienza en el período temprano, como indirectamente, después de que las fibras nerviosas alcanzan el músculo efector.

PALABRAS CLAVE: Experimento; Lesión del nervio periférico; Complejo neuromuscular; Electroestimulación a largo plazo; Morfología.

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