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Correlation analysis of electroneuromyographic, functional-anatomical and morphometric indicators of regeneration of the injured sciatic nerve

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Objective. To study the electrophysiological, functional-anatomical and morphometric indicators of the sciatic nerve regeneration after complete transection and connection of the nerve stumps with epineural sutures and adhesives.

Materials and methods. The experiments were carried out on white outbred male rats. The efficiency of sciatic nerve regeneration was investigated with the standard 4-6 epineural sutures and the use of polyethylene glycol hydrogel Duraseal or Tisseel fibrin glue with 2 fixation sutures. On the 14th, 30th and 60th days after the complete transection of the sciatic nerve and subsequent connection of the nerve stumps with epineural sutures, hydrogel or fibrin glue, limb function was assessed using SFI test and ENMG. The distal segment of the nerve was sampled for electron microscopic and morphometric studies. The density of the regenerated myelinated nerve fibers was studied and a correlation analysis was performed with the results of SFI test and ENMG.

Results. The use of adhesives provides a similar result of connecting the transected sciatic nerve, as in standard neuroorrhaphy, and regeneration of myelinated nerve fibers in the distal nerve. The regeneration density of myelinated nerve fibers significantly increased on the 30th and 60th days in the groups in which Duraseal hydrogel and Tisseel fibrin glue were used, without a statistically significant difference in ENMG parameters (M-response amplitude, nerve conduction velocity, latency period) and the result of SFI test. The amplitude of M-response on the 30th day was statistically significantly lower after the connection of nerve stumps with Tisseel fibrin glue in comparison with the Duraseal hydrogel, whereas on the 60th day there was no difference according to the results of electrophysiological studies.

Conclusions. The efficiency of sciatic nerve regeneration after the combined connection exceeds the standard technique, and the results of pathophysiological assessments are more often correlated with morphometry data on the 30th day.

Key words: peripheral nerve injury; neuroorrhaphy; adhesive joint; Duraseal; Tisseel; regeneration

Introduction

Regeneration of a traumatically damaged peripheral nerve remains an urgent neurosurgical problem. Nerve repair at the structural and functional level is often insufficient, especially in large nerve defects [1]. Regeneration processes range from wallerian degeneration and regeneration through the damage area to muscle reinnervation. The efficiency of these processes is influenced not only by the nature and size of the defect, but also by the method of microsurgical restoration [2,3]. The proposed biodegradable adhesive gels are considered to be a potential alternative to the suture connection of the nerve stump. It is assumed that additional adhesion of the injured nerve stump (with hydrogel based on Duraseal polyethylene glycol or Tisseel fibrin glue) will allow fewer

sutures, which will reduce the trauma of microsurgical repair and is potentially associated with better functional recovery.

Under experimental conditions, the restoration of the injured nerve is studied mainly by the methods of histology and morphometry, and the conclusion about the result of the recovery is based on the morphometric assessment of the distal segment of the nerve. Functional assessment of peripheral nerves in animals is methodologically more complex and is based on electroneuromyography (ENMG) or contradictory (in our opinion) results of quantitative assessment of limb function (e.g., SFI-test) [4]. The analysis of the obtained data gives a better idea of neuromuscular recovery, and the use of correlation analysis expands the arsenal of statistical methods of

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data evaluation, although the estimated parameters do not always correlate with each other [4,5].

Objective: to investigate the electrophysiological, functional anatomical and morphometric indicators of sciatic nerve regeneration after complete transection and connection of nerve stumps with epineural suture and adhesives.

Materials and methods

The study was performed on white outbred male rats aged 5-6 months. Average body weight was (250 ± 25) g. Rats were kept in the vivarium of the Romodanov Neurosurgery Institute, Ukraine. There were 5 animals in each plastic box (container) with a lattice under standard light regime, free access to water and granular complete feed.

Bioethics

The experiments were performed in compliance with bioethics standards specified in the EU Directive 2010/63/EU "On the protection of animals used for scientific purposes" (1986), the European Convention for the Protection of Vertebrate Animals used for Experimental and Scientific Purposes (Strasbourg, 18.03.1986), the Law of Ukraine dated February 21, 2006 No 3447-IV "On protection of Animals from Cruelty" (2006). The study design was approved by the Bioethics Committee of Bogomolets National Medical University (Meeting Minutes No.113).

Characteristics of groups

Rats were divided into experimental groups:

- 1) "C" (control) - intact rats (n=5);
- 2) "PO" - pseudo-operated rats were given access to the sciatic nerve without transection (n=15);
- 3) "ES" - rats were simulated the complete transection of the sciatic nerve and the nerve stumps with 4-6 epineural sutures were connected (n=15);
- 4) "PEG" - rats were simulated the complete transection of the sciatic nerve and the nerve stumps with 2 epineural sutures and hydrogel based on polyethylene glycol (Duraseal) were connected (n=15);
- 5) "FG" - rats were simulated the complete transection of the sciatic nerve and the nerve stumps with 2 epineural sutures and fibrin glue were connected (Tisseel) (n=15).

Animals of each group were sacrificed on the 14th, 30th and 60th day after simulation of sciatic nerve injury, 5 rats at each observation period (that is, each group consisted of 3 subgroups of 5 animals, which were randomly divided into subgroups).

Study design

The model of complete transection of the sciatic nerve is used. The surgery was performed under general anesthesia (xylazine ("Sedazin", "Biowet", Poland was administered intraperitoneally at a dose of 15 mg / kg body weight and ketamine ("Calypsol", "Gedeon Richter A.O.", Hungary) at a dose of 70 mg / kg of body weight. A linear skin incision along the lateral surface of the thigh in the projection of the sciatic nerve on the left was made, the sciatic nerve was mobilized, it was transected perpendicularly, followed by end-to-end fixation by epineural neuroorrhaphy, atraumatic needle with monofilament polyamide suture No 10/0, 4-6 epineural

sutures were applied using an operating microscope (× 12). In the "PEG" and "FG" groups, nerve stumps were fixed with gels and two additional "fixation" epineural sutures were applied at a distance of 180°. After careful hemostasis in all groups of animals layered suturing of the postoperative wound was performed using an atraumatic needle with a monofilament polyamide suture No 4/0. In all animals, after simulating the complete transection of the sciatic nerve, severe paresis of the limb was noted (the main criterion for involving animals in experimental groups). No lethal outcomes were recorded in the experiment.

To prevent infectious complications, a solution of benzylpenicillin was injected subcutaneously in the posterior cervical region at a dose of 1 million IU / kg of body weight. For antiinflammatory and antiedematous therapy a solution of dexamethasone at a dose of 6 mg / kg of body weight was administered intraperitoneally. Animals were sacrificed by administering a lethal dose of xylazine and ketamine.

Electrophysiological and functional anatomical studies were performed before sacrificing the rats from the experiment. After that, the sciatic nerve was sampled for electron microscopic and morphometric studies.

Conducting the Sciatic Functional Index test (SFI). The limbs of rats were labeled with fucorcin and the animals were sent to the test track. Limb fingerprints were collected, the length between the limb fingers was measured and quantitative values were calculated using the formula [6].

Electroneuromyography. Before sacrificing animals were anesthetized (intraperitoneally, xylazine at a dose of 15 mg / kg of body weight and ketamine at a dose of 70 mg / kg of body weight), were fixed on the operating table belly down. A grounding electrode was fixed along the tail (a metallized tape moistened with 0.9% sodium chloride solution, 20 mm wide, 100 mm long), the sciatic nerve was isolated from the pelvic outlet to its distribution into branches, and the operating field was cleaned with saline. The nerve was covered with a bipolar platinum hooked electrode (monopolar diameter - 0.22 mm, the distance between monopolars - 5.5 mm). Stimulation current was generated by a digital electroneuromyograph "Neuro-MVP-Micro" (LLC "Neurosoft", Russia), was supplied in pulse mode (pulse duration - 5 ms) with a frequency of 0.2 Hz (1 pulse for 5 s) with a step of increasing the current strength 1 mA. The excitation was recorded by electroneuromyography using a concentric needle electrode (length - 25 mm, diameter - 0.3 mm, withdrawal area - 0.015 mm²) at the motor point of the calf muscle. The distance between the stimulation and registration electrodes was about 25 mm. The function of neuromuscular recovery was assessed by ENMG parameters: M-response amplitude (AmV), latent M-response period (ms), nerve conduction velocity (NCV) (mm / ms). The amplitude of the maximum M-response, obtained in most cases - at a stimulation current of 3 mA was only analyzed.

Morphological and morphometric studies. The sciatic nerve was excised and placed in a 10% solution of neutral formalin, the distal fragment was additionally fixed in a 2.5% solution of glutaraldehyde in phosphate buffer with 1% osmium tetrachloride. From the sciatic

nerve (which contained the proximal segment, suture area and distal segment), frozen sections of 15 μm thick were made in a cryostat and impregnated with silver nitrate to study the direction and features of nerve fiber regeneration [7]. The distal segment of the nerve (in transverse projection) after fixation in osmium tetrachloride was dehydrated (ethanol, acetone) and embedded in a mixture of epoxy resins Epon-Araldite. Semi-thin sections of the nerve (<2 μm) were stained with toluidine blue [8]. They were examined under a light microscope (Olympus BX-51). Each nerve was photographed at $\times 1000$ and using the software (Carl Zeiss (AxioVision SE64 Rel.4.9.1) the average density of myelinated nerve fibers was evaluated (microphoto size is 216 \times 138 μm (0.03 mm^2), the number of photos per sample - 10 -15 (2/3-3/4 of nerve cross-section (units per 1 mm^2)) The average diameter (μm) of myelinated nerve fibers was estimated as the mean value of the large and small diameters of a single fiber. Ultrathin sections were examined using an electron microscope Tescan Mira 3 LMU (Czech Republic) in transmission mode (STEM).

Statistical analysis

Statistical data processing was performed using StatPlus software (version 7.0 Microsoft). The normal data distribution in comparison groups was determined by the Kolmogorov - Smirnov test. All data are presented as the arithmetic mean and the standard error of mean ($M \pm m$). The probability of difference between comparison groups was determined by one-way ANOVA test with Bonferroni post-hoc test. Spearman's rank correlation coefficient (r_s) was used to determine the relationship between the group-averaged parameters. The results were considered statistically significant with the probability of null hypothesis $p < 0,05$.

Results and discussion

The dynamics of regeneration in the sciatic nerve after complete transection was characterized by an increase in the density of nerve fibers in the area of the nerve suture and their invasion in the distal segment of the nerve. There was a statistically significant increase in the density of nerve fibers between the 14th and 60th days, remyelination of axial cylinders and elimination of introduced hydrogels in the "PEG" and "FG" groups. On the 60th day, the focal accumulations of "PEG" were encapsulated with connective tissue, and fibrin glue was completely eliminated. In the three main groups, the density and remyelination of nerve fibers in the distal segment of the nerve increased on the 30th and 60th day compared to the 14th day (**Fig. 1,2**).

To determine the quantitative indicators of nerve regeneration, the morphometry of the distal nerve was conducted on semi-thin sections in the transverse projection of the nerve section. In the "ES" group the number of myelinated nerve fibers in the distal nerve increased between the 30th and 60th day, and the level of regeneration on average was 12.6, 18.8 and 21.5 % respectively, on the 14th, 30th and 60th day (**Table 1**). In the "PEG" group a more rapid regeneration of myelinated nerve fibers was found, a statistically significant difference was found between the indicators on the 14th and 30th day, the level of regeneration was 15.2, 24.2 and 29.4%. In the "FG" group the dynamics of regeneration in the distal nerve was similar to that in the "PEG" group, the level of

regeneration was 15.8, 28.3 and 32.1%. A statistically significant difference between the "PEG" and "ES", "FG" and "ES" groups was established on the 30th and 60th day, that is the dynamics of regeneration of myelinated nerve fibers in the "PEG" and "FG" groups was more significant.

Changes in ENMG parameters consisted in an increase in the amplitude of M-response throughout the follow-up period. In the "ES" group, a statistically significant increase in the amplitude of M-response of the calf muscle compared to the indicators on the 14th and 30th days was recorded on the 60th day (see **Table 1**). There was no difference in this indicator compared to the "C" group. The nerve conduction velocity in all periods of the experiment was lower than in the "C" group. On the 60th day, a smaller NCV was detected compared to the 14th day and a longer latency period of M-response, which correlated with the level of regeneration ($r_s=0.90$, $p=0,04$ and $r_s=-1.00$, $p < 0,01$) (**Table 2**). This can be explained by an increase in the regeneration of "thin" weakly myelinated nerve fibers.

In the "PEG" group. the amplitude of M-response increased statistically significantly on the 60th day. The nerve conduction velocity in the period from the 14th to the 30th day approached the rate of the "C" group. and on the 60th day - decreased. The latency period did not differ statistically significantly from the index of intact animals. ENMG parameters did not differ from those of the "ES" group. When analysing the correlation between the average number of myelinated nerve fibers and ENMG parameters a strong correlation was found on the 30th day with the amplitude of M-response ($r_s=0.90$, $p=0.04$) and NCV on the 14th day ($r_s=0.90$, $p=0.04$). At other periods. Spearman's test did not reach statistically significant values.

In the "FG" group. a statistically significant increase in the amplitude of M-response was registered relative to the indicator on the 14th day. but on the 30th day the indicator was lower than the values in the "PEG" group. The nerve conduction velocity in the period from the 14th to the 30th day did not differ from the reference (group "C"). and on the 60th day it decreased with the background of increasing the latency period of velocity conduction. According to the results of correlation analysis. a strong correlation was found between the level of nerve regeneration and the amplitude of M-response ($r_s=0.90$, $p=0.04$). NCV ($r_s=0.90$, $p=0.04$) on the 30th day. At other periods. Spearman's test did not reach statistically significant values.

The dynamics of recovery was characterized by an improvement in the locomotor function of rats limb according to the results of SFI test (see **Table 1**). There was no statistically significant difference in the dynamics of recovery (SFI change) between the "ES", "PEG" and "FG" groups. Although in the "FG" group the SFI values on the 60th day did not differ from those on the 30th day. in the "ES" and "PEG" groups a significant recovery of function was observed at all periods. Spearman rank correlation analysis revealed a strong connection between the average number of myelinated nerve fibers in the distal nerve and SFI in the "ES" group on the 14th and 30th day. in the "PEG" group - on the 14th and 60th day. in the "FG" group - on the 30th day. At other periods. the results of correlation analysis were not statistically significant.

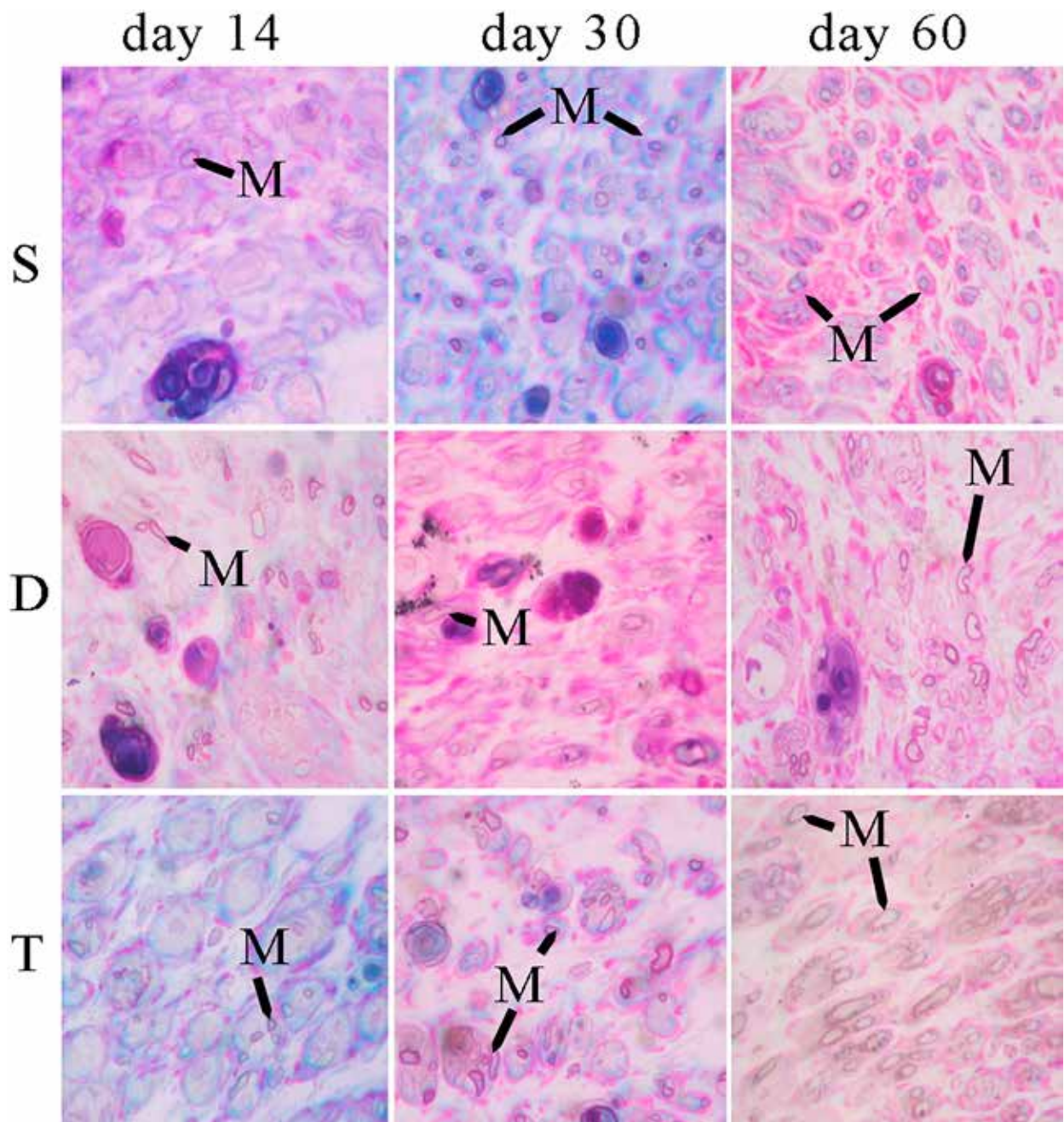


Fig. 1. Micrographs of semi-thin sections of the distal segment of the sciatic nerve after complete section and connection of the nerve stumps: S - epineural suture; D - Duraseal hydrogel; T - Tisseel fibrin glue; M - myelin nerve fiber. The density and diameter of nerve fibers are increased on the 30th and 60th days. Staining with toluidine blue and basic fuchsin $\times 1000$

The obtained data of morphometry of regenerated nerve. ENMG and functional test. correlations between functional parameters and the level of regeneration expand the understanding of the nature of structural and functional recovery of the neuromuscular apparatus. For example, the intensity of regeneration in the injured nerve increases statistically significantly on the 60th day with standard neurorrhaphy and is characterized by higher values of the amplitude of M-response during this period, which indicates an increase in the number of operational units. Compared with standard neurorrhaphy, in the hydrogel group, nerve regeneration, according to morphometry, began earlier, showing a

strong correlation with an increase in the amplitude of M-response on the 30th day. Decreased NCV and increased latency period can be attributed to two factors. Firstly, regenerative growth of nerve fibers continues, in particular weakly myelinated ones, which reinnervate the paretic muscle. Secondly, within 60 days, the paretic muscle transforms into a complex of peripheral parts of rather heterogeneous motor units, with different temporal characteristics of electrical excitability, which stretches in time and makes it difficult to detect the onset of the integral electrical response of the muscle – the M-response. Under such conditions, an increase in the central part of M-response (its amplitude) with

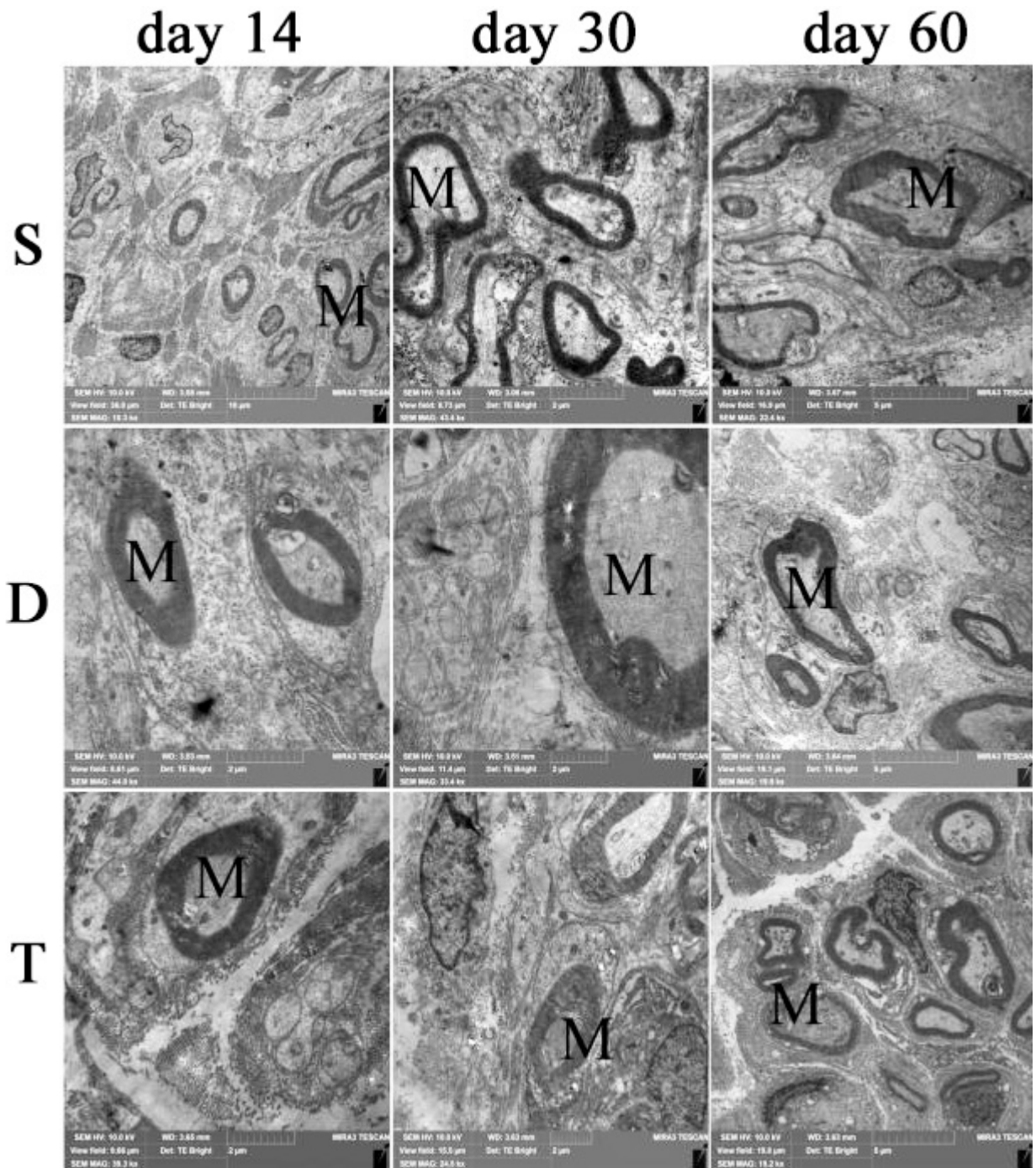


Fig. 2. Electron diffraction patterns of the distal segment of the sciatic nerve after complete section and connection of the nerve stumps: S - epineural suture; D - Duraseal hydrogel; T - Tisseel fibrin glue; M - myelin nerve fiber. Remyelination of nerve fibers, nerve fibers regenerate in clusters.

the background of a decrease in NCV may be associated with the synchronization of excitation of structurally preserved muscle fibers with successful reinnervation [9] and the coverage of a large number of muscle fibers with fewer motor neurons. Registered M-responses at early stages of nerve regeneration are often asynchronous, and the higher amplitude and synchronized ENMG parameters are recorded in 3 and 5 months after neuroorrhaphy [10].

With a statistically significant increase in the number of regenerating nerve fibers in the distal nerve. ENMG

parameters in the "PEG" and "FG" groups did not differ from those in the "ES" group. This indicates a peculiar course of regeneration process under such conditions. Probably, under conditions of microsurgical nerve restoration a critical number of functioning operating units is formed rather quickly. It can provide the restoration of muscle contractile function and additional regeneration of nerve fibers is not accompanied by a significant increase in motor function. A more pronounced recovery of ENMG parameters in the "PEG"

Table 1. Morphometric, electrophysiological and functional studies outcomes in rats after complete sciatic nerve transection and connection of the nerve stump in various ways

Group	Duration, day	Number of myelinated nerve fibers	Amplitude of the M-response, mV	Nerve conduction velocity, mm/ms	Latency period, ms	SFI
C	14	5703.6±256.0	19.30±0.95	40.90±1.61	0.68±0.02	-2.3±0.2
	30					-3.0±0.3
	60					-2.4±0.3
PO	14	5643.4±209.4	20.70±1.39	37.60±3.11	0.78±0.02	-3.1±0.3
	30	5734.4±211.4	19.10±1.78	44.40±3.42	0.62±0.04**	-2.3±0.2
	60	5445.7±177.3	18.70±1.07	41.1±2.54	0.65±0.02	-3.0±0.2
ES	14	720.6±48.5*	3.93±0.84*	31.60±1.98*	0.84±0.03	-70.3±2.4*
	30	1076.8±61.7*	6.67±1.19*	30.60±1.40*	0.85±0.05	-35.5±1.5*.*
	60	1229.8±55.8*.*	13.30±1.27*.*.#	25.40±1.79*.*#	1.12±0.08*.*.#	-26.3±1.4*.*.#
PEG	14	868.1±39.7*	3.86±0.31*	30.40±3.02	0.91±0.04*	-70.0±0.8*
	30	1384.7±77.6*.*.*^	7.28±0.75*	37.90±2.94	0.76±0.06	-32.0±1.3*.*
	60	1675.9±72.8*.*.*^	15.00±3.05*.*.#	30.00±0.98*	0.83±0.03	-24.3±1.4*.*.#
FG	14	905.4±40.2*	3.02±0.29*	36.80±2.97	0.91±0.08*	-70.4±2.2*
	30	1618.8±121.7*.*.*^	5.10±0.29*.*#	33.90±1.09	0.76±0.02	-42.7±1.8*.*
	60	1829.8±60.1*.*.*^	11.20±2.29*.*.*.#	31.10±3.22*	1.28±0.18*.*.*.#&	-36.3±2.3*.*

Note. The difference is statistically significant (p < 0.05) compared with the indicator: * - «C» and «PO» groups; ^ - «ES»group; & - «PEG» group, ** - on the 14th day; # - on the 30th day.

Table 2. Correlation analysis outcomes between the number of myelinated nerve fibers in the distal nerve, ENMG parameters, the result of the functional test of the limb of rats and the connection of the nerve stumps in various ways

Indicator	Group	Duration, day	Amplitude of the M-response	Nerve conduction velocity	Latency period	SFI
Number of myelinated nerve fibers in the distal nerve	ES	14	r _s =0.50; p=0.39	r _s =0.90; p=0.04	r _s =-0.20; p=0.75	r _s =0.90; p=0.04
		30	r _s =0.30; p=0.62	r _s =0.90; p=0.04	r _s =-0.50; p=0.39	r _s =0.90; p=0.04
		60	r _s =-0.70; p=0.19	r _s =0.90; p=0.04	r _s =-1.00; p<0.01	r _s =0.70; p=0.19
	PEG	14	r _s =0.50; p=0.39	r _s =0.90; p=0.04	r _s =-0.40; p=0.50	r _s =0.90; p=0.04
		30	r _s =0.90; p=0.04	r _s =0.70; p=0.19	r _s =-0.80; p=0.10	r _s =0.70; p=0.19
		60	r _s =0.30; p=0.62	r _s =0.70; p=0.19	r _s =-0.30; p=0.62	r _s =0.90; p=0.04
	FG	14	r _s =-0.60; p=0.28	r _s =0.10; p=0.87	r _s =0.82; p=0.09	r _s =-0.60; p=0.28
		30	r _s =0.90; p=0.04	r _s =0.90; p=0.04	r _s =-0.70; p=0.19	r _s =0.90; p=0.04
		60	r _s =0.60; p=0.28	r _s =0.60; p=0.28	r _s =-0.60; p=0.28	r _s =0.60; p=0.28

group compared to the "FG" group indicates a more significant functional recovery in the first group.

The functional state of the limb after neuroorrhaphy of the damaged nerve is of great importance. One of the tests that allows assessing the function of the limb is SFI [11]. Many experimental studies use SFI to assess recovery. Its effectiveness has been demonstrated.

Usually the SFI value correlates with the morphometric data of the regenerated nerve, as well as with the results of ENMG [3]. In our studies, the definition of SFI allowed us to state the positive dynamics of the function of the paretic limb throughout the follow-up period. It is important that the final result of the regeneration process does not depend on the technique of connecting

the stumps of the transected nerve. However, in the "FG" group, in contrast to the "ES" and "PEG" groups, the period of significant SFI increase was limited to the first month of the post-traumatic period, which is consistent with the conclusion of low NCV values in this group on the 60th day compared with the "PEG" group. Other studies have found similar recovery dynamics with SFI test, starting from the 14th day after sciatic nerve injury. The dynamics of recovery when using Duraseal hydrogel was statistically significantly better than that when using fibrin glue [12]. It should be noted that the data obtained relate to the short but most intense period of traumatic and regenerative process, as well as the fact that the methodology for assessing motor function using SFI-test is irrelevant for the number of reasons during the first 3 weeks after transection of the sciatic nerve [13]. Therefore, an additional analysis of electrophysiological data and their correlation with the level of nerve regeneration makes it possible to eliminate the drawbacks of assessing the recovery at the early stages of nerve regeneration. Usually, a strong correlation between the level of nerve regeneration and electrophysiological parameters or the result of SFI test was detected on the 30th, 14th or 60th day. Throughout the follow-up periods, a strong correlation with high statistical significance was found for NCV and slightly weaker – according to the results of SFI-test in the "ES" group. Lower values of the correlation coefficient in the "PEG" and "FG" groups (except for the follow-up period of 30 days, when a strong correlation was statistically confirmed) should also be considered as the effect of the applied means on recovery, since the analysis of M-response amplitude revealed a statistically significant difference at various periods of the experiment.

Conclusions

1. The intensity of nerve fiber regeneration in the sciatic nerve after complete transection in the "PEG" and "FG" groups exceeded that in the "ES" group.
2. A significant increase in the amplitude of M-response was noted only on the 60th day. Its value in the "PEG" and "FG" groups did not differ from that in the "ES" group. On the 30th day, this indicator was statistically significantly correlated with the number of myelin fibers in the distal part of the nerve.
3. In the dynamics of follow-up, there was no change in the NCV in the experimental groups. The value of the indicator correlated with the number of myelin fibers in the distal part of the nerve in the "ES" group throughout the follow-up periods, in the "PEG" group - on the 14th day, in the "FG" group - on the 30th day.
4. Throughout the follow-up period, the correlation between the number of myelin fibers in the distal part of the nerve and the latency period in the "PEG" and "FG" groups was absent, in the "ES" group – it was noted only on the 60th day.
5. The value of SFI test in the "ES" and "PEG" groups correlated with the number of myelin fibers in the distal part of the nerve. In the "FG" group, a significant increase in SFI test result was observed during the first month. On the 14th and 60th day, there was no correlation between the result of SFI test and the number of myelin fibers in the distal part of the nerve.

Disclosure

Conflict of interest

The authors declare no conflict of interest.

Ethical standards

All procedures performed on experimental animals comply with the norms and ethics and are approved by the ethics committee of the scientific institution where the study was conducted.

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The study was performed without sponsorship.

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