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Histomorphometric assessment of the tibial nerve and small muscles of the foot after internal neurolysis and autogenous plastic surgery of the tibial portion of the sciatic nerve in rats

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Abstract

Introduction Transformations of the nerves and muscles of the distal extremities after nerve injuries at the level of the proximal segments are critical for the restoration of functions, but have not been sufficiently studied in experimental biological models. **Purpose** Histomorphometric evaluation of the tibial nerves and plantar interosseous muscles after internal neurolysis and autogenous plasty of the tibial portion of the sciatic nerve (SN) in rats. **Materials and methods** The study was performed on 21 male Wistar rats, aged 8-10 months. Series 1 (internal SN neurolysis) – n = 6. Series 2 (autogenous SN neuroplasty) – n = 8. Control – 7 intact rats. The rats were euthanized 6 months after the operation. Light microscopy and histomorphometry of transverse semithin sections of the tibial nerve at the level of the middle third of the lower leg and paraffin sections of the plantar interosseous muscles of the foot were performed. **Results** In series 1, endoneural vessels had increased diameters and wall thickness in the tibial nerve, but a smaller lumen compared to the norm; the dimensional characteristics of the myelinated fiber population were increased due to myelin decompactization and axonal edema; about 10 % of myelinated fibers were destructively changed. In series 2, the numerical density of the endoneural vessels of the tibial nerve was doubled in comparison with the intact one; the numerical and dimensional composition of the regenerated myelinated fibers indicated active but incomplete regeneration. The vascularization index of the plantar interosseous muscles in series 1 was close to normal, in series 2 it decreased twice, the median of muscle fiber diameters was reduced by 12.41 % ($p = 0.000$) and 20.96 % ($p = 0.000$), respectively. Muscle fibers with a diameter of more than 30 μm increased in series 2 compared to series 1. **Conclusion** Internal neurolysis and interfascicular autoplasty of the sciatic nerve cause multidirectional changes in the endoneural vessels of the tibial nerve, which predetermine the multidirectional nature and severity of denervation and reinnervation changes in the nerves of the lower leg and small muscles of the foot.

Keywords: sciatic nerve, internal neurolysis, autogenous plasty, tibial nerve, interosseous muscles of the foot, histomorphometry

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INTRODUCTION

Injuries to the nerves of the extremities occur in 2.8 to 10% [1, 2] of trauma patients. Such patients require longer hospitalization and rehabilitation than those with injuries without nerve damage [3], since denervation of muscles and integumentary tissues causes loss of movement and sensory disturbances [4], what significantly reduces the quality of life of patients.

The severity of nerve injuries varies from slight compression to complete interruption of all its structures [5]. It determines the outcomes and tactics of treatment. Even in complete peripheral nerve interruption, nerve fibers can spontaneously regenerate through a small defect or scar [6], but in most cases this does not lead to restoration of functions. Therefore, surgical revision and reconstruction of nerve fiber bundles using internal neurolysis and interfascicular autoplasty are indicated in the absence of signs of restoration of a partially damaged nerve and in complete anatomical interruptions of the nerves, [7].

Internal neurolysis is used as a preparatory stage for interfascicular autoplasty, but if the continuity of nerve fiber bundles is retained, it acts as a self-sufficient operation [8, 9, 10]. However, data on the effect of internal neurolysis on reinnervation in compressive neuropathy in the experiment and in the clinic are contradictory [11-14], which actualizes a targeted experimental study of the effect of internal neurolysis of an intact nerve on the state of its distal branches and innervated muscles.

Most experimental studies of neuroregeneration were conducted on the sciatic nerve of rats [15]. Their relevance and clinical significance is determined by the high incidence of sciatic nerve iatrogenic injuries and in combat trauma [16-20]. However, in the available literature, we did not find data on the effect of internal neurolysis and interfascicular autoplasty of the sciatic nerve on the state of the nerves of the lower leg and small muscles of the foot in experimental animals. Since slow regenerative growth of axons, delaying muscle reinnervation, reduces the efficiency of functional recovery [21], the condition of the distal extremities in case of nerve injuries at the level of the proximal segments is especially critical for functional recovery.

Purpose To conduct histomorphometric evaluation of the tibial nerves and plantar interosseous muscles after internal neurolysis and autogenous plasty of the tibial portion of the sciatic nerve in rats.

MATERIALS AND METHODS

The study was performed on 21 laboratory male Wistar rats aged 8-10 months (weight 360-420 g). Animals were kept in two-story cable cages with a smooth bottom and bedding of wood shavings under controlled hygienic conditions. They had free access to water and standard food. The experiment was carried out in accordance with the European Convention for the Protection of Vertebrate Animals, Directive 2010/63/EU of the European Parliament and the Council of the European Union for the protection of animals used for scientific purposes, and SP 2.2.1.3218-14; GOST 33217-2014; GOST 33215-2014. The design of the study was approved by the ethics board of the institution (protocol No. 2 (57) dated May 17, 2018). Fourteen rats were included in two experimental series. In experimental series, xylazine hydrochloride and tiletamine/zolazepam (0.8 mg and 0.4 mg per 100 g of rat weight, respectively) were injected intramuscularly before the operation, and the skin hair was cut off in the region of the right thigh and lower leg. In the operating room, the skin was treated with iodine-alcohol tincture. In series 1 (internal neurolysis), an extra-projection approach to the right sciatic nerve at the level of the middle third of the thigh was performed using an acute-blunt method through the biceps femoris muscle. Under an operating microscope (OPMI-6, Germany), using a sharp vascular probe and iridectomy scissors, the tibial portion of the sciatic nerve was exposed by epifascicular longitudinal epineurotomy for 2 cm, after which the wound was sutured in layers. In series 2, after a similar approach to the nerve and its division into bundles, a 6-mm-long tibial portion was resected. Next, using microsurgical techniques (magnification of the operating microscope 8-16×, suture material of 9-0), the resected area was sutured in situ. The control series consisted of 7 intact rats (age range 16-18 months, corresponding to the age of the operated rats at the time of their euthanasia 6 months after the operation).

The tibial nerves were excised, subjected to aldehyde-osmium fixation, and embedded in araldite. Semi-thin sections (thickness 0.5-1.0 μm) were produced using diamond knives on a Nova LKB ultramicrotome (Sweden), stained with methylene blue and basic magenta. Plantar interosseous muscles in the region of the 3rd metatarsal bone, innervated by the plantar nerve, which is a branch of the tibial nerve, were fixed in 4% formalin solution, decalcified in a mixture of hydrochloric and formic acids, dehydrated in ethanol, and embedded in paraffin. Paraffin sections (5-7 μm) were produced on a Reichert microtome (Austria), stained with Masson's three-color method, hematoxylin and eosin. Microscopic study and digital imaging were performed using an AxioScope.A1 microscope and an AxioCam digital camera (Carl Zeiss MicroImaging GmbH, Germany).

The VideoTesT Master-Morphology, 4.0 software was used to determine the average diameters of myelin fibers (a sample of 400-500 fibers in each rat), their axons, the G coefficient (the ratio of the axon diameter to the fiber diameter), the thickness of the myelin sheath, the proportion of altered myelin nerve fibers (in %), average diameters of endoneural microvessels, their lumens, wall thickness and the modified Kernogan index (the ratio of the diameter of the lumen to the thickness of the vascular wall), the numerical densities of myelin fibers and endoneural microvessels per 1 mm^2 of the section area were calculated. Histograms of the distribution of myelin fibers by diameters were built (1 μm step). Using the Zen blue software (Carl Zeiss MicroImaging GmbH, Germany), full-color images of paraffin transverse sections of the plantar interosseous muscles at x400 magnification were used to measure the average diameter of each muscle fiber (D, μm); an average of 200 fibers from one case was analyzed. Histograms of the distribution of fibers by diameters were built (10 μm step). The numerical density of microvessels and muscle fibers was determined, and the vascularization index (Iv) was calculated.

Statistical data processing was performed using the computer software Attestat, version 9.3.1 (developer I.P. Gaydyshev, certificate of registration with Rospatent No. 2002611109). Due to significant differences in the distribution of some samples from the normal one, the data in tables were presented as medians (Me) and quartiles (Q1; Q3). To test statistical hypotheses about differences in pairwise comparison of experimental series with each other and with the norm, the Chi-square test, the Mann-Whitney test were used, and the Smirnov test was used to identify differences in distribution functions.

RESULTS

Microscopic study of transverse semi-thin sections of the sciatic and tibial nerves of series 1 rats (internal neurolysis) showed that the majority of myelin fibers, as in intact nerves, had a normal structure; some parts of fibers featured either demyelinization or thickening, defibration or uneven coloring of the myelin. Some fibers had deformation and darkening of axons (Fig. 1 a, b). In series 2 (autoneuroplasty), in the graft of the tibial portion of the sciatic nerve, in the tibial portion distal to the graft, and also in the tibial nerve at the level of the

middle third of the leg, regenerated myelinated nerve fibers were smaller compared to the norm and series 1; some fibers formed regeneration clusters (Fig. 1 c). In series 2, fibroblasts, macrophages, and mast cells were more common in the endoneurium along with nucleated profiles of myelinated and unmyelinated nerve fibers.

Analysis of the quantitative parameters of the population of myelinated fibers of the tibial nerve in series 1 (Table 1) revealed a decrease in the median of their numeric density compared to the norm by 11.78 % (p = 0.041). The proportion of reactive-destructively altered fibers in series 1 significantly exceeded the norm by 3.19 % (p = 0.003). The median diameter of myelin fibers in series 1 increased by 28.26 % (p = 0.002), the median axon diameter increased by 9.22 % (p = 0.000), the median thickness of myelin sheaths increased by 18.63 % (p = 0.000)

In series 2, the differences from the norm had the opposite direction. The numeric density of myelin conductors exceeded the norm by 34.36 % (p = 0.001) with a significant decrease in the percentage of altered fibers relative to the norm by 2.70 % (p = 0.000). The median diameters of myelin fibers were reduced in comparison with the norm by 52.00 % (p = 0.000), the median diameters of axons were reduced by 51.05 % (p = 0.000) and the thickness of myelin sheaths decreased by 52.94 % (p = 0.000) (Table 1).

Analysis of endoneural vascularization of the tibial nerve (Table 2) showed that in series 1 and 2, the numerical density of endoneural vessels increased relative to the norm by 5.61 % (p = 0.806) and by 90.23 % (p = 0.028), respectively; their diameters increased by 21.15 % (p = 0.001) and 15.42 % (p = 0.001), the wall thickness increased by 33.93 % (p = 0.000) and 17.10 % (p = 0.003), respectively. However, the lumen diameters in series 1 decreased relative to the norm by 13.92 % (p = 0.018), but in series 2 increased by 29.75 % (p = 0.069). In series 2, the Kernogan index did not differ from the norm, and in series 1, due to thickening of the walls of microvessels and a decrease in the diameter of their lumens, it decreased by 41.43 % (p = 0.000) (Table 2), what indicates a significant deterioration in their conductive capabilities.

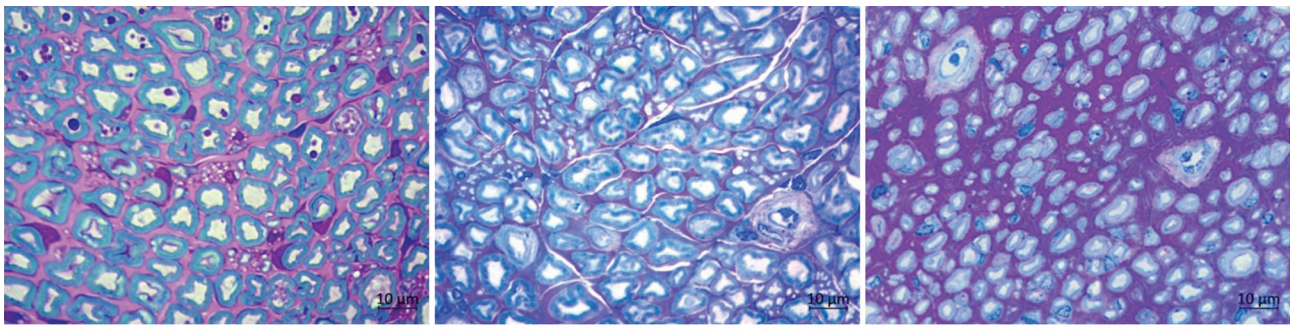

Fig. 1 Fragments of semi-thin transverse epoxy sections of the tibial nerves of rats: *a* intact nerve; *b* series 1 (internal neurolysis); *c* series 2 (autoneuroplasty). Stained +with methylene blue, azure II and basic magenta, ×500

Table 1

Numerical and dimensional characteristics of myelinated nerve fibers of the tibial nerve of rats in the intact norm and experimental series Me (Q1; Q3)

Parameter	Norm (intact animals) n = 7	Series 1 (internal neurolysis) n = 6	Series 2 (autoneuroplasty) n = 8
Numerical density of myelin fibers (per 1 mm^2)	15040 (12859; 15499) p^{n-1} = 0.041*; p^{n-2} = 0.001*	13261 (11825; 14237) p^{1-2} = 0.000*	20207 (18140; 22618)
Proportion of altered myelin fibers (%)	6.75 (5.70; 8.13) p^{n-1} = 0.003*; p^{n-2} = 0.000*	9.94 (7.82; 10.68) P^{1-2} = 0.000*	4.05 (2.38; 5.06)
Myelin fiber diameter (µm)	6.73 (5.50; 8.75) p^{n-1} = 0.002*; p^{n-2} = 0.000*	7.35 (5.41; 8.51) p^{1-2} = 0.000*	3.50 (2.79; 4.04)
Diameter of axons of myelin fibers (µm)	4.34 (3.54; 5.18) p^{n-1} = 0.000*; p^{n-2} = 0.000*	4.74 (3.50; 5.64) p^{1-2} = 0.000*	2.42 (1.84; 2.84)
Myelin thickness (µm)	1.02 (0.72; 1.30) p^{n-1} = 0,000*; p^{n-2} = 0,000*	1.21 (0.87; 1.53) p^{1-2} = 0.000*	0.54 (0.44; 0.62)

Notes: p^{n-1} – significance of differences in pairwise comparison of the norm and series 1; p^{n-2} – norm and series 2; p^{1-2} – series 1 and 2 according to the Mann-Whitney test; * – differences are significant at $p \leq 0.05$

Table 2

Numerical and dimensional characteristics of endoneural microvessels of the tibial nerve of rats in the intact norm and experimental series Me (Q1; Q3)

Parameter	Norm (intact animals) n = 7	Series 1 (internal neurolysis) n = 6	Series 2 (autoneuroplasty) n = 8
Numerical density of endoneural microvessels (per 1 mm^2)	63.94 (63.00; 74.94) $p^{n-1} = 0.806$; $p^{n-2} = 0.028$*	67.53 (65.99; 67.07) $p^{1-2} = 0.027$*	121.63 (97.79; 130.56)
Microvessel diameter (μm)	12.39 (9.31; 15.51) $p^{n-1} = 0.001$*; $p^{n-2} = 0.001$*	15.01 (12.04; 18.13) $p^{1-2} = 0.511$	14.30 (11.91; 18.18)
Diameter of lumen (μm)	4.74 (3.28; 6.36) $p^{n-1} = 0.018$*; $p^{n-2} = 0.069$	4.08 (0.00; 6.34) $p^{1-2} = 0.013$*	6.15 (3.60; 8.52)
Vessel wall thickness (μm)	3.86 (2.59; 4.66) $p^{n-1} = 0.000$*; $p^{n-2} = 0.003$*	5.17 (4.46; 6.23) $p^{1-2} = 0.001$*	4.52 (3.48; 5.66)
Kernogan index	1.40 (0.94; 1.95) $p^{n-1} = 0.000$*; $p^{n-2} = 0.904$	0.82 (0.00; 1.38) $p^{1-2} = 0.000$*	1.46 (0.89; 1.98)

Notes: p^{n-1} – significance of differences in pairwise comparison of the norm and series 1; p^{n-2} – norm and series 2; p^{1-2} – series 1 and 2 according to the Mann-Whitney test; * – differences are significant at $p < 0.05$

An analysis of the distribution of myelin fibers by diameters (Fig. 2) showed that in series 1 the distribution graph was similar to that of the intact rat tibial nerve: the distribution was bimodal, and although the right mode and plotting were generally shifted by 1 class towards larger diameters, the distribution function had no statistically significant differences from the norm (Fig. 2). In series 2, in contrast to the norm and series 1, the distribution was unimodal, the base of the graph is shortened by 3-4 digits, and the mode is shifted to the left towards smaller diameters, the distribution function significantly differed from both the norm and series 1 (Fig. 2).

Microscopic examination of the plantar interosseous muscles of the third metatarsal bone innervated by the tibial nerve in series 1 showed pronounced fibrosis of the perimysium, and fibrosis of the peri- and endomysium in series 2 (Fig. 3). In contrast to the intact muscles, where sections of muscle fibers had a polygonal shape, in both experimental series there were myocytes with rounded or angular contours, altered tinctorial properties – a decrease in color intensity in the central areas (Fig. 3 a). In the nerve trunks located in the connective tissue sheaths of the muscles, axonal and Wallerian degeneration of myelinated nerve fibers was observed. Reactive-destructive changes in muscle and nerve fibers were more pronounced in series 2.

In both experimental series, an increase in the number of intramuscular nuclei was noted; along with hypertrophied fibers, atrophic myons were encountered, as well as myons at different stages of necrotic death (Fig. 4).

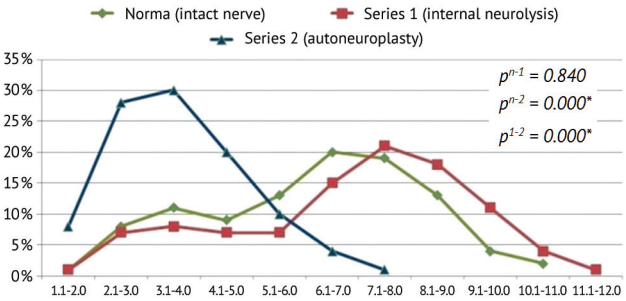

Fig. 2 Graphs of the distribution of myelinated nerve fibers of the tibial nerve of rats in normal and experimental series by diameter (step – 1 μm). The abscissa axis is the diameter (μm), the ordinate axis is the proportion of fibers (%). Significance levels of differences in pairwise comparison of the norm (n), series 1 (1) and series 2 (2) according to the Smirnov criterion – p^{n-1}, p^{n-2}, p^{1-2}; * – differences are significant at $p < 0.05$

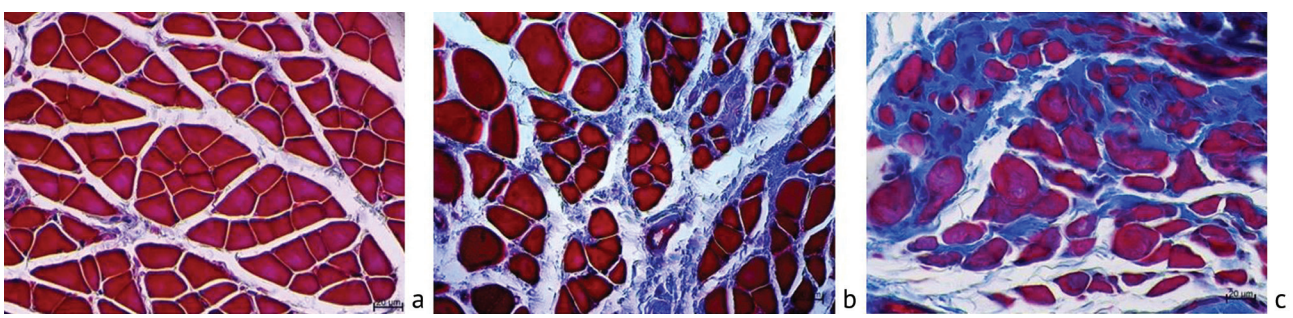

Fig. 3 Fragments of transverse paraffin sections of the plantar interosseous muscles: *a* norm (intact muscle); *b* series 1 (internal neurolysis); *c* series 2 (autoneuroplasty). Transverse paraffin section, Masson tricolor staining, ×400

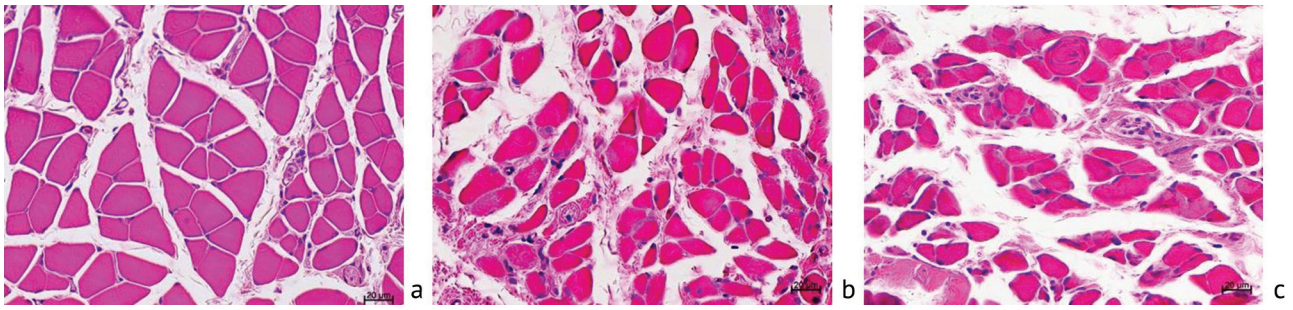

Fig. 4 Fragments of transverse paraffin sections of plantar interosseous muscles: *a* norm (intact muscle); *b* series 1 (internal neurolysis); *c* series 2 (autoneuroplasty). Transverse paraffin section, stained with hematoxylin and eosin, ×400

Analysis of the quantitative indicators of muscle fibers revealed a decrease in the median diameters of their diameters in series 1 and 2 relative to the norm by 12.41 % (p = 0.000) and 20.96 % (p = 0.000), respectively. In series 2, the median of the muscle fiber vascularization index was reduced twice (Table 3).

In the intact norm, the diameters of muscle fibers did not exceed 70 μm; the distribution by diameters was unimodal; the number of histogram classes was 7 (Fig. 5). Muscle fibers with a diameter of 21-30 μm (53.9%) dominated; small fibers with a diameter of up to 10 μm and large fibers with a diameter of 31-40 μm accounted for 6% and 1%, respectively; fibers with a diameter of more than 40 μm were rare (Fig. 5). In the experimental series, the histogram peak shifted one class to the left (11-20 μm diameter muscle fibers dominated). The number of histogram classes in series 1 was 4 (from less than 10 μm to 31-40 μm) and in series 2 it was 6 (from less than 10 μm to 51-60 μm). In series 2, compared with the norm and series 1, the fraction of muscle fibers with a diameter of up to 10 μm increased by 3.5 times, and the portion of muscle fibers with a diameter of 21-30 μm significantly decreased; there were single muscle fibers with a diameter of more than 40 μm (Fig. 5). Differences in the distribution function of muscle fibers in series 1 from the norm were statistically insignificant; series 2 significantly differed from series 1 and from the norm.

Table 3

Quantitative characteristics of muscle fibers of the plantar interosseous muscles of rats in normal and experimental series Me (Q1; Q3)

Parameter	Norm (intact animals), n = 7	Series 1 (internal neurolysis), n = 6	Series 2 (autoneuroplasty), n = 8
Fiber diameter (μm)	21.52 (17.13; 25.31) $p^{n-1} = 0.000$*; $p^{n-2} = 0.000$*	18.85 (15.24; 22.41); $p^{1-2} = 0.000$*	17.01 (11.43; 20.52)
Index of fiber vascularization	1.00 (0.54; 1.00) $p^{n-1} = 0.050$; $p^{n-2} = 0.040$*	1.00 (0.69; 1.00); $p^{1-2} = 0.000$*	0.50 (0.40; 0.80)

Notes: p^{n-1} – level of significance of differences in pairwise comparison of the norm and series 1; p^{n-2} – the norm and series 2; p^{1-2} – series 1 and 2 according to the Mann-Whitney test; * – differences are significant at $p < 0.05$.

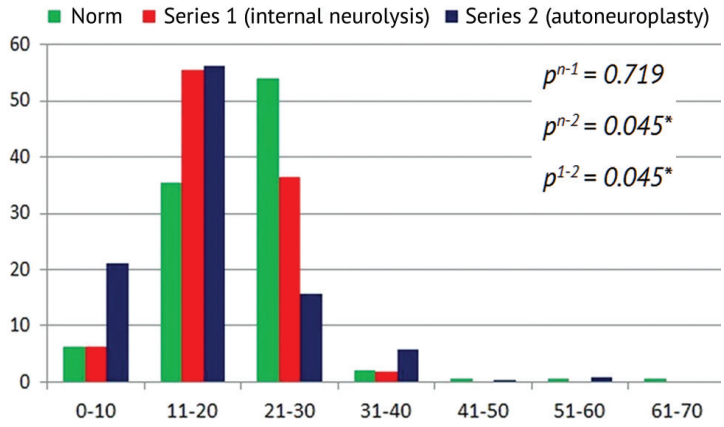

Fig. 5 Histograms of the distribution of muscle fibers of the plantar interosseous muscles of rats in the norm and experimental series by diameter (step – 10 μm). The abscissa axis is diameters (μm), the ordinate axis is the fraction of fibers (%). Significance levels of differences in pairwise comparison of the norm (n), series 1 (1) and series 2 (2) according to the Smirnov criterion – p^{n-1}, p^{n-2}, p^{1-2}; * – differences are significant at $p < 0.05$

DICUSSION

In this study, the impact of internal neurolysis and autoplasty of the sciatic nerve on vascularization and the number and size composition of the fiber population of the tibial nerve and small muscles of the foot was evaluated for the first time.

Previously, other authors in experiments with internal neurolysis of the tibial nerve in rabbits revealed not only fibrosis of the nerve sheaths, but also damage to some part of the nerve fibers, despite the fact that the barrier function of the perineurium and endoneural vessels, according to the authors, was not impaired [22]. In our study, it was found that internal neurolysis of the sciatic nerve of rats at the level of the middle third of the thigh which is inevitably accompanied by damage to the network of epineural vessels, leads to significant changes in the endoneural vessels of the tibial nerve. Compared to the vessels of the tibial nerve of intact rats, they become larger, thicker, but they have a smaller lumen and, as a result, a reduced throughput. Probably, it results in ischemia of the endoneurium and the population of myelinated fibers of the tibial nerve changes. Six months after surgery, a significant part of them (about 10%) was destructively changed, and many had decompacted myelin sheaths and edematous axons. Myelin decompactization and axonal edema in the optic nerve of rats were previously described by other authors and interpreted as manifestations of chronic degeneration in ischemic neuropathy [23]. In our experiments, the sparing model of internal neurolysis [24] included epifascicular epineurotomy without dissection of the perineurium. However, six months after the operation, fibrosis of the perimysium as well as qualitative and quantitative signs of denervation changes in muscle fibers were expressed in the plantar interosseous muscles of the foot innervated by the tibial nerve.

According to Goth D [25], external neurolysis in experimental animals did not cause changes in the normal mosaic pattern of muscle fibers, while after internal neurolysis, the phenomenon of muscle fiber grouping was expressed. This indicated not only the presence of a denervation/reinnervation process and the possibility of recovery, but, above all, collateral reinnervation [26]. In our study, we did not reveal the phenomenon of muscle fibers grouping in either the first or second series of experiments; however, the index of vascularization of the plantar interosseous muscles in the series with internal neurolysis was close to normal, the differences in the distribution function of muscle fibers in diameter from intact muscles were statistically insignificant, but the median diameters were significantly reduced in comparison with the norm.

In the experiment with series 2, internal neurolysis of the sciatic nerve was performed as the first stage of the operation, the exposure of the tibial portion. After resection of the tibial portion and suturing of the resected fragment in situ, two zones of sutures and a non-vascularized graft were located on the way of regenerating axons. According to other authors, vascularized grafts provide faster regeneration, but the end result is comparable to non-vascularized ones [27], since non-vascularized grafts revascularize over time mainly from the proximal coaptation of the operated nerve [28]. Moreover, the newly formed blood vessels are considered not only as a factor in the survival of cell and tissue structures but also as a pathway for the migration of Schwann cells during neuroregeneration [29].

Our study established for the first time that autoneuroplasty of the rat sciatic nerve promotes a pronounced hypervascularization of the endoneurium of the tibial nerve, as evidenced by a twofold increase in the numerical density of endoneural vessels in comparison with the intact nerve. In contrast to the series with internal neurolysis, the endoneural vessels of the tibial nerve in the animals after autoneuroplasty of the sciatic nerve had a less thickened wall, wide gaps, and a capacity comparable to the norm. The tibial nerve was neuroticized by a large number of regenerated myelinated fibers; however, their numerical and dimensional composition testified to the incompleteness of the regeneration process. The index of vascularization of the muscles reinnervated by the tibial nerve reduced by one half; fibrosis of the perimysium and endomysium, qualitative and quantitative changes in muscle fibers indicated insufficient reinnervation. However, a greater representation of muscle fibers with a diameter of more than 30 μm in the second series compared to the first one indicated the possibility of further recovery. Until now, it is not known whether the vascularization of the regenerating nerve and reinnervated muscles can be considered as prognostic parameters [30], but our study and other studies in similar areas significantly expand the existing ideas about possible targets for neuroregenerative effects.

Thus, the authors have developed a new biological model, microsurgical autoneuroplasty of the tibial portion of the sciatic nerve of the Wistar rat, which will allow further trials of new low-traumatic intraoperative neuroregenerative and revascularizing effects aimed at improving the results of treatment of restorative and reconstructive operations on the nerves of the extremities.

CONCLUSION

Internal neurolysis and interfascicular autoplasty of the sciatic nerve have caused multidirectional changes in the endoneural vessels of the tibial nerve that predetermine the nature and severity of denervation and reinnervation changes in the nerves of the lower leg and small muscles of the foot.

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