BASIC RESEARCH

Beneficial effects of treadmill training in experimental diabetic nerve regeneration

Tais Malysz,[I] Jocemar Ilha,[I] Patrícia Severo do Nascimento,[I] Kátia De Angelis,[II] Beatriz D'Agord Schaan,[I,III] Matilde Achaval[I]

[I] Universidade Federal do Rio Grande do Sul - Programa de Pós-Graduação em Neurociências, Departamento de Ciências Morfológicas, Instituto de Ciências Básicas da Saúde, Porto Alegre, Rio Grande do Sul, Brazil. [II] Universidade São Judas Tadeu - Laboratório do Movimento Humano, São Paulo, São Paulo, Brazil. [III] Universidade Federal do Rio Grande do Sul - Serviço de Endocrinologia, Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil.

OBJECTIVES: We investigated the effects of treadmill training (10 weeks) on hindlimb motor function and nerve morphometric parameters in diabetic rats submitted to sciatic nerve crush.

MATERIALS AND METHOD: Wistar rats (n = 64) were divided into the following groups: non-diabetic; trained non-diabetic; non-diabetic with sciatic nerve crush; trained non-diabetic with sciatic nerve crush; diabetic; trained diabetic; diabetic with sciatic nerve crush or trained diabetic with sciatic nerve crush. Diabetes was induced by streptozotocin injection (50 mg/kg, iv). Hindlimb motor function was evaluated weekly by assessing sciatic functional indices, and the proximal and distal portions of the sciatic nerve were used for morphometric analysis.

RESULTS: At 13 weeks post-injury, the distal nerve portion of all injured groups and the proximal nerve portion of the diabetic with sciatic nerve crush group presented altered morphometric parameters such as decreased myelinated fiber diameter ($\sim 7.4 \pm 0.3\mu m$ vs $\sim 4.8 \pm 0.2\mu m$), axonal diameter ($\sim 5 \pm 0.2\mu m$ vs $\sim 3.5 \pm 0.1\mu m$) and myelin sheath thickness ($\sim 1.2 \pm 0.07\mu m$ vs $\sim 0.65 \pm 0.07\mu m$) and an increase in the percentage of area occupied by endoneurium ($\sim 28 \pm 3\%$ vs $\sim 60 \pm 3\%$). In addition, in the non-diabetic with sciatic nerve crush group the proximal nerve portion showed a decreased myelinated fiber diameter ($7.4 \pm 0.3\mu m$ vs $5.8 \pm 0.3\mu m$) and myelin sheath thickness ($1.29 \pm 0.08\mu m$ vs $0.92 \pm 0.08\mu m$). The non-diabetic with sciatic nerve crush, trained non-diabetic with sciatic nerve crush, diabetic with sciatic nerve crush and trained diabetic with sciatic nerve crush groups showed normal sciatic functional index from the 4th, 4th, 9th and 7th week post-injury, respectively. Morphometric alterations in the proximal nerve portion of the diabetic with sciatic nerve crush and non-diabetic with sciatic nerve crush groups were either prevented or reverted to values similar to the non-diabetic group by treadmill training.

CONCLUSION: Diabetic condition promoted delay in sciatic nerve regeneration. Treadmill training is able to accelerate hindlimb motor function recovery in diabetic injured rats and prevent or revert morphometric alterations in proximal nerve portions in non-diabetic and diabetic injured rats.

KEYWORDS: Diabetes; Sciatic nerve crush; Motor function; Nerve morphometry; Treadmill training.

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E-mail: achaval@ufrgs.br

Tel.: 55 51 3308-3624

INTRODUCTION

Peripheral neuropathy is a common complication in patients with diabetes mellitus and consists of several clinical syndromes that affect motor, sensory and autonomic nerves. Usual pathologic alterations are axonal atrophy, demyelination, nerve fiber loss and disordered neural repair.[1]

Streptozotocin (STZ)-induced diabetes is a well-established animal model for diabetes mellitus and experimental diabetic neuropathy in rats; however, these animals show minimal nerve fiber loss in peripheral nerves.[2,3] Accordingly, a useful animal model for studying nerve fiber regeneration in diabetic neuropathy is the combination of surgically-induced nerve injury with STZ-induced hyperglycemia in rats.[4]

Moreover, nerve regeneration in diabetes is essential for the reversal of peripheral neuropathy and also promotes the recovery of nerves from injury as a result of acute nerve compression and entrapment. However, none of the therapeutic procedures used to prevent progression of nerve dysfunction and promote nerve fiber regeneration were able to completely restore neural function.[4]

Walking training is generally indicated by medical professionals in the treatment of the diabetic patients.

However, data concerning the effectiveness of this type of regular exercise in the treatment of human diabetic peripheral neuropathy is scarce.[5]

Previous studies have shown that treadmill exercise training can improve peripheral nervous tissue regeneration in non-diabetic rats and mice after nerve injury[6-8]. In diabetic rats, this training modality can improve the morphologic features and increase the size of A cells from the L5 dorsal root ganglion[9], and improve autonomic nerve dysfunction in diabetic rats.[10,11] Although swimming exercise training has been shown to have protective and therapeutic effects on diabetic experimental peripheral neuropathy[12], there are no data on the effectiveness of treadmill training in the regeneration of nerves affected by experimental diabetes.

Thus, the aim of this study was to investigate the effects of treadmill training on hindlimb motor function recovery and the morphological parameters of nerves in diabetic rats submitted to sciatic nerve crush.

MATERIALS AND METHODS

Experimental design

Experiments were performed on sixty four, 12-week-old, male Wistar rats, weighing 260 g to 315 g, from a local breeding colony (ICBS, Universidade Federal do Rio Grande do Sul, Brazil). The rats were housed in standard plexiglass boxes, under a 12 h light/dark cycle, in a temperature-controlled environment (20±1℃), with food and water available *ad libitum*. The animals were cared for in accordance with Brazilian law and the recommendations of the Brazilian Society for Neurosciences, Review Committee of the School of Veterinary Surgery, University of Buenos Aires and the International Brain Research Organization (IBRO), which are in compliance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (publication no. 85-23, revised 1985).

The animals were randomly assigned into groups as follows: non-diabetic (N, n = 6); trained non-diabetic (TN, n = 6); non-diabetic with sciatic nerve crush (NC, n = 6); trained non-diabetic with sciatic nerve crush (TNC, n = 6); diabetic (D, n = 6); trained diabetic (TD, n = 8); diabetic with sciatic nerve crush (DC, n = 9) or trained diabetic with sciatic nerve crush (TDC, n = 7). During the course of the 17 weeks of the experimental protocol, of the 40 diabetic rats, 10 rats died.

Diabetes induction

After fasting (6 h), rats were rendered diabetic by a single intravenous STZ injection (50 mg/kg, Sigma Chemical Co, St Louis, MO, USA) diluted in citrate buffer (pH 4.5; 2 mL/kg). Non-diabetic rats were only injected with citrate buffer. After 6 h of fasting, glycemia was evaluated using test strips (Advantage, Roche, Indianapolis, IN, USA) at 48 h after diabetes induction, weekly after the beginning of the experimental period and also 24 h after the last bout of exercise. Only those rats with glycemic levels >300 mg/dL were maintained in the diabetic groups. No insulin therapy was used during the study.

Surgical Procedures

Four weeks after diabetes induction (Fig. 1), the animals were anesthetized using ketamine and xilazine (90 and 15 mg/kg, i.p., respectively; Vetbrands, Brazil), and the right sciatic nerve was exposed by splitting the gluteal muscle, and crushed immediately behind the emergence of the lower limit of the gluteus maximus muscle. The crush was made with 1 mm non-serrated hemostatic forceps for 30 seconds[8] and the crush site was marked by a fine suture through the edge of the epineurium. In rats from the groups without crush (N and D), the sciatic nerve was exposed, but not crushed. The muscles were re-approximated, the skin was closed with 4-0 nylon sutures and the animals were maintained in their cages for 2 weeks.

Maximal Exercise Test

Two weeks after the surgery, all animals were adapted on a treadmill for 10 min at 0.3 km/h for 4 days (Fig. 1). Maximal exercise tests were performed (MET) after the adaptation period (MET1), at the end of the 5th week (MET2) and at the end of the training (MET3) to determine, adjust and compare the efficacy of the treadmill training protocol, respectively. The test consisted of a graded exercise on the treadmill, with speed increments of 0.3 km/h every 3 minutes, starting at 0.3 km/h and continuing up to the maximal intensity attained by each rat.[13]

Training Program

Treadmill training was performed at low to moderate intensity (40-50% maximal running speed of the MET). Training began in the 4th week after sciatic nerve crush (Fig. 1), with two sessions per day (at least 4 hours between bouts), 5 days per week for 10 weeks adapted from De Angelis et al.[10] In the sessions, the rats ran for 10 min in the 1st week, attained 40 min at the end of the 4th week and 60 min at the end of the 7th week.

Analysis of Hindlimb Motor Function

Following sciatic nerve crush and until the conclusion of the treadmill training program, all animals were subjected

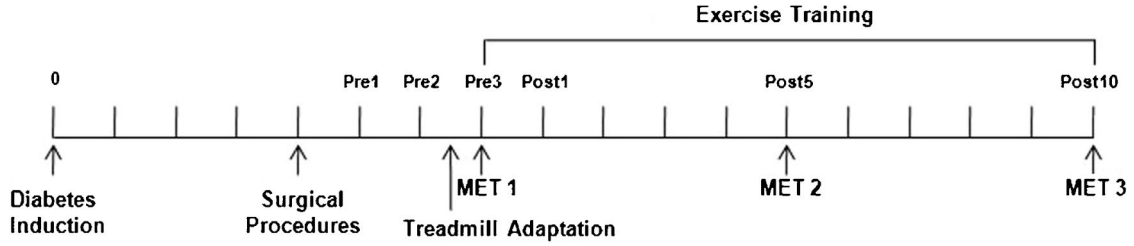

Figure 1 - Time course line showing the 17 weeks of the experimental procedures and weeks used to assess motor activity after surgical procedures before beginning the training program (pre 1-3 weeks) and after each week of the training period (post 1-10 weeks). MET 1; maximal exercise test before training; MET 2; maximal exercise test during training; MET 3; maximal exercise test at the end of the training period.

to a series of weekly motor activity assessments. These assessments were held before initiation of the exercise training protocol (pre 1-3 weeks) and after each week of the exercise training period (post 1-10 weeks), making a total of 13 assessments for each rat (Fig. 1). Recovery of locomotor activity was considered proof of adequate post nerve crush re-innervation of the right hindlimb, and functional recovery was monitored by analysis of the free-walking pattern. This method describes an index based on measurements of the footprints of walking rats, which provides a reliable and easily quantifiable method of evaluating the functional condition of the sciatic nerve[14]. For this test, the rats were trained to walk over a white sheet of paper covering the bottom of a 100 cm-long, 8.5 cm-wide track, which ended in a dark box. Afterwards, the animals had their plantar hind feet painted with dark dye and were then placed on the track to walk.

The rat footprints were used to determine the following measurements: distance from the heel to the third toe [print length (PL)]; distance from the first to the fifth toe [toe spread (TS)]; and distance from the second to the fourth toe [intermediary toe spread (ITS)]. These three measurements were obtained from the experimental (E) and normal (N) sides. Several prints of each foot were obtained on each track, but only three prints of each foot were used to determine the mean measurements in the E and N sides. These mean measurements were then included in the sciatic function index-formula: SFI = − 38.3 (EPL - NPL) / NPL + 109.5 (ETS - NTS) / NTS + 13.3 (EIT - NIT) / NIT − 8.8.[14]

The result obtained was considered a functional index of the sciatic nerve, where 12 to -12 represents excellent function; -13 to -37, good function; -38 to -62, average function; -63 to -87, unsatisfactory function; -88 to -112, complete deficit; and -113 to -137, worse than complete deficit.[15]

Histological and Morphometric nerve studies

Twenty-four hours after the end of the exercise training protocol, the rats were anesthetized with sodium thiopental (50 mg/kg; i.p.; Cristalia, Brazil) and then transcardially perfused using a peristaltic pump (20 mL/min, Milan, Brazil) with 0.9% saline solution followed by a fixative solution containing 2.5% glutaraldehyde (Sigma Chemicals Co.) and 4% paraformaldehyde (Reagen, Brazil) in 0.1 M phosphate buffer (PB), pH 7.4, at room temperature. The right sciatic nerve was isolated and excised in 2 short segments (~3 mm), one taken 5 mm before, and one after, the crush injury site (proximal and distal portions, respectively). The portions were then post-fixed in 1% OsO_4 (Sigma Chemicals Co) in PB, dehydrated in a graded series of alcohol and propylene oxide (Electron Microscopy Science, Hatfield, PA, USA), embedded in resin (Durcupan, ACM-Fluka, Buchs, Switzerland) and polymerized at 60° C for 72 h. Afterward, cross-sectional semithin sections (900 nm) were obtained using an ultramicrotome (Leica Ultracut UCT 2.0, Vienna, Austria) and stained with 1% toluidine blue (Merck, Darmstadt, Germany) in 1% sodium tetraborate (Ecibra, Brazil).

Images of the proximal and distal portions of the right sciatic nerve obtained from the 6 rats per group were captured and digitalized (initially 1000× and then further amplified 200% for analysis) using a Nikon Eclipse E-600 microscope (Nikon, Tokyo, Japan) coupled to a digital camera and Image Pro Plus Software 6.0 (Media Cybernetics, Silver Spring, MD, USA). For morphometric evaluation, a set of 6 images from each nerve portion was chosen by a blinded examiner using random sampling of one slice, 3 random images from the periphery and 3 random images from the center of the nerve.[8] The total area of the sciatic segment examined was the sum of the 6 randomly selected areas (8919.36 μm^2 in total). Both proximal and distal portions of the right sciatic nerves were analyzed separately, and the average number of fibers analyzed per nerve segment was 155. The morphometrical measurements included the myelinated fiber density (number of myelinated fibers/mm^2), average myelinated fiber diameter (μm), average axonal diameter of the myelinated fiber (μm), average myelin sheath thickness (μm), percentage of area occupied by myelinated fibers (%) and percentage of area occupied by the endoneurium (%). The latter measurement included the unmyelinated fibers and degenerative debris.

The estimate of the myelinated fiber density was determined by examining the ratio of the fibers/total area analyzed. The myelin sheath thickness was estimated using the measurement tools from the Image Pro Plus Software. To estimate the axonal and fiber diameters, the area of each individual fiber was measured and the value obtained was converted to the diameter of a circle with an equivalent area. The area sizes were estimated using a point-counting technique,[8] employing grids with point density of 1 point per 1.54 μm^2 and the equation: $A = \Sigma p.a/p$, where A is area, Σp the total of counted areas/point and a/p the area/point value (1.54 μm^2). By adding together all the myelinated fiber areas it was possible to arrive at an estimate of the total area occupied by myelinated fibers and calculate its percentage from the total analyzed area (100%). By deducting this percentage of area from the total analyzed area it was possible to estimate the percentage of area occupied by the endoneurium.

Statistical analysis

Glycemic levels, body weight, maximal speed evaluations, SFI values and morphologic measurements were analyzed using repeated measures analysis of variance (ANOVA). The Bonferroni test was used to adjust the results of the multiple comparisons at $P<0.05$. Descriptive data were expressed as means ± SEM (standard error of the mean). Data were run on SPSS® 11.5 (Statistical Package for the Social Sciences, Inc., Chicago, IL, USA).

RESULTS

Glycemic levels, body weight and maximal exercise test

During the entire experimental period, the diabetic groups (D, TD, DC, TDC) presented higher glycemic levels and lower body weight when compared to non-diabetic groups (N, TN, NC, TNC; $P<0.05$; Table 1). In both the diabetic and non-diabetic groups glycemic levels remained unchanged throughout the experiment ($P>0.05$). At the end of the experiment, whilst non-diabetic groups presented a gain in body weight vs their baseline values ($P<0.05$), diabetic groups showed maintenance ($P>0.05$) of their body weight. At the end of the study, there were no differences in terms of glycemic levels or body weight among the members within either the diabetic or non-diabetic groups ($P>0.05$; Table 1).

Table 1 - Glycemic levels, body weight and maximal exercise test (MET) in non-diabetic (N), trained non-diabetic (TN), diabetic (D), trained diabetic (TD), non-diabetic submitted to sciatic nerve crush (NC), trained non-diabetic submitted to sciatic nerve crush (TNC), diabetic submitted to sciatic nerve crush (DC) and trained diabetic submitted to sciatic nerve crush (TDC) groups.

	Glycemic levels (mg/dL)		Body Weight (g)		MET (km/h)		
Groups	Initial	Final	Initial	Final	MET1	MET2	MET3
N	93±4	92±2	281±8	368±10[b]	1.08±0.1	1.08±0.1	0.96±0.1
TN	95±2	89±2	269±18	360±18[b]	1.08±0.1	1.44±0.1[ce]	1.74±0.1[cde]
D	401±28[a]	412±15[a]	266±5	290±3[a]	0.88±0.2	0.96±0.2	0.96±0.2
TD	446±37[a]	466±8[a]	288±12	274±5[a]	0.9±0.1	1.2±0.1[cef]	1.56±0.1[cdef]
NC	98±1	97±2	292±6	393±13[b]	0.9±0.1	0.9±0.1	0.84±0.1
TNC	91±4	88±2	293±12	352±19[b]	1.08±0.2	1.5±0.2[ce]	1.86±0.1[cde]
DC	396±16[a]	456±20[a]	267±9	252±9[a]	0.6±0.1[ef]	0.9±0.1 [c]	0.9±0.1[c]
TDC	445±27[a]	475±17[a]	260±7	261±13[a]	0.6±0.1[ef]	1.02±0.1[cef]	1.38±0.1[cdef]

Data are reported as mean ± SEM.
[a]$P<0.05$ *vs.* N, TN, NC and TNC groups
[b]$P<0.05$ *vs.* initial in the same group
[c]$P<0.05$ *vs.* MET 1 in the same group
[d]$P<0.05$ *vs.* MET 2 in the same group
[e]$P<0.05$ *vs.* corresponding MET of the sedentary groups (N, D, NC, DC)
[f]$P<0.05$ *vs.* corresponding MET of the non-diabetic trained groups (TN and TNC).

As expected, the trained groups (TN, TD, TNC and TDC) presented a progressive increase in their exercise capacity, as verified by the increase in the maximum speed of running in the METs over the training period ($P<0.001$); however this increase was lower in trained diabetic groups when compared to trained non-diabetic groups ($P<0.05$) (Table 1). There were no differences between MET performance in N, D and NC groups over time. However, sciatic nerve crush induced a decrease in physical capacity in the diabetic group, as observed by the reduction in the maximum speed of running in the MET1, as compared to the MET2 and MET3 in the DC group ($P<0.05$; Table 1).

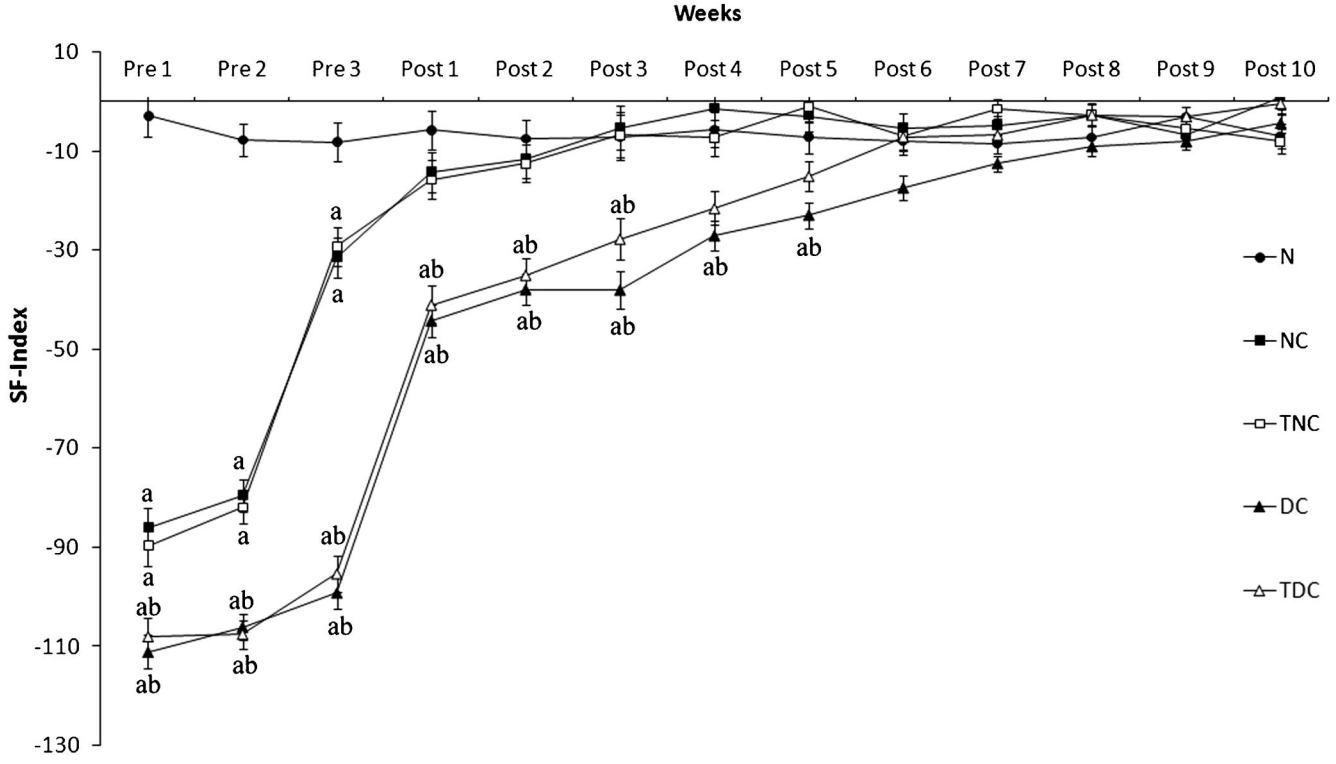

Figure 2 - Graphs showing functional recovery (sciatic functional index, SFI) before beginning the training program (pre 1-3 weeks) and after each week of the training period (post 1-10 weeks) in non-diabetic (N, n = 6), non-diabetic with sciatic nerve crush (NC, n = 6), trained non-diabetic with sciatic nerve crush (TNC, n = 6), diabetic submitted to sciatic nerve crush (DC, n = 9) and trained diabetic submitted to sciatic nerve crush (TDC, n = 7) groups. a $P<0.05$ *vs.* N group; b $P<0.05$ *vs.* NC and TNC groups. Data are expressed as means ± SEM.

Sciatic Functional Index

The SFI values, including pre-training (pre-1 to 3) and post-training week records (post-1 to 10), are shown in Figure 2. As expected, in the uninjured groups (N, TN, D and TD), all SFI values were normal, remaining stable at around -11 throughout the experiment (data not shown). There were no differences in SFI values between these groups ($P>0.05$).

Right footprints (ipsilateral to the sciatic nerve crush) of the injured groups (NC, TNC, DC and TDC) presented alterations in relation to the results obtained in left footprints, which included an increase in the print length value and a decrease in the toe spread and intermediary toe spread values. These alterations affected the SFI values, which tended to be more negative after sciatic nerve crush, a finding which indicates loss of motor function.

In the pre-training evaluation (pre-1 to 3), sedentary and trained non-diabetic injured rats (NC and TNC) showed significantly lower SFI values than the uninjured group ($P<0.05$, Fig. 2). These differences were not seen during all the subsequent post-training weeks ($P>0.05$). No differences were noted between the SFI values in the NC and TNC groups ($P>0.05$).

Throughout the 17 weeks of this study, no differences in SFI values ($P>0.05$) were observed between the DC and TDC groups. However, while the sedentary diabetic injured rats (DC) had lower SFI values when compared to the uninjured groups (N, TN, D and TD) and injured non-diabetic groups (NC and TNC) from pre-training week 1 until the post-training week 5 ($P<0.05$), the trained diabetic injured rats (TDC) had lower SFI values (vs uninjured groups and injured non-diabetic groups) from pre-training week 1 until the post-training week 3 ($P<0.05$). Therefore, the TDC group showed functional recovery two weeks before of the DC group. In addition, in subsequent weeks (after post-training week 5 for the DC group and after post-training week 3 for the TDC group) no differences were seen between any of the groups ($P>0.05$).

Histological studies

Analysis of the digitized images from the proximal and distal portions of the sciatic nerve in the uninjured rats (N,

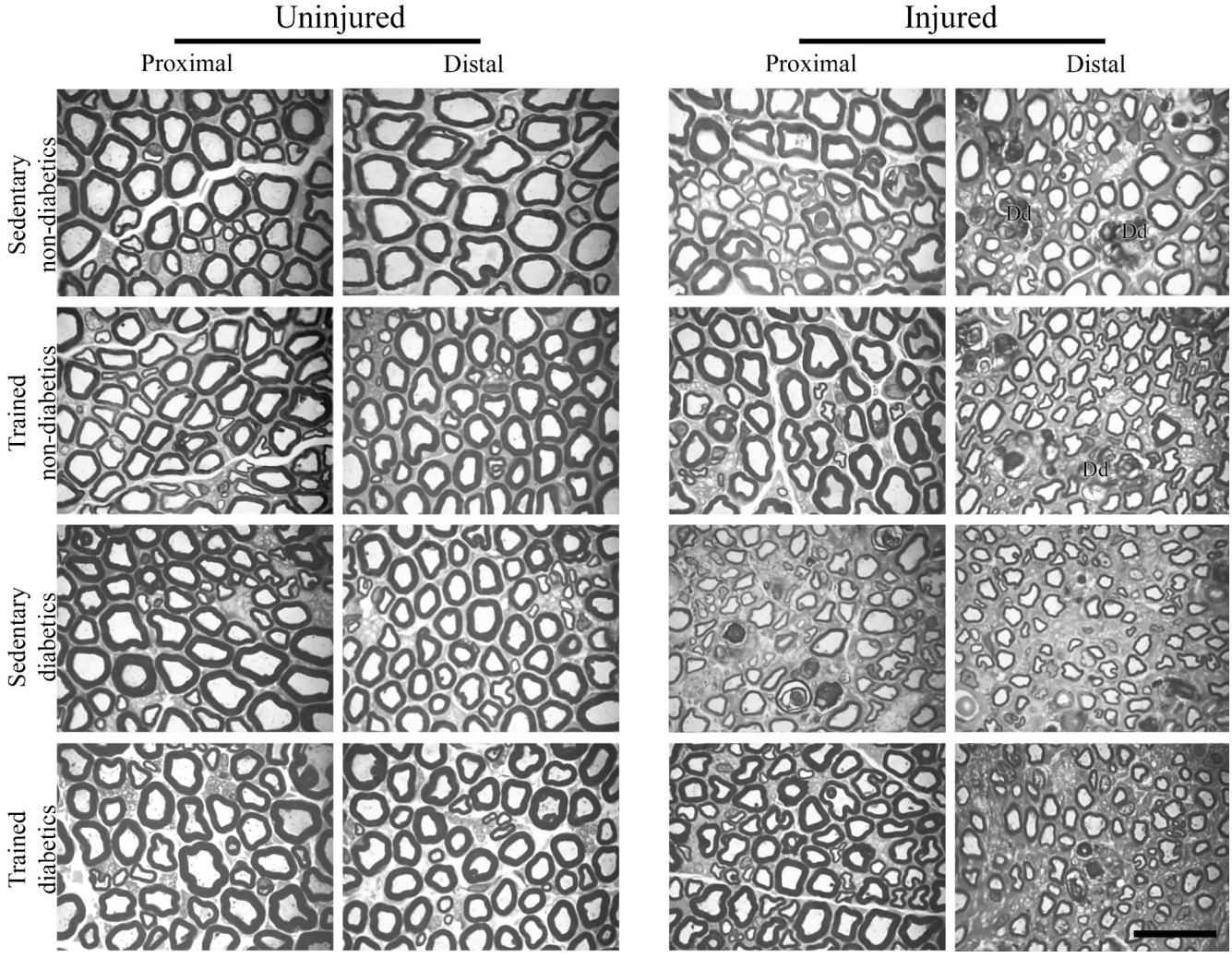

Figure 3 - Digitized images of semithin cross-sections obtained from the proximal and distal portions of the sciatic nerve from different groups in this study. Note the predominance of small thin myelinated fibers, enlargement of the space occupied by endoneurial tissue and presence of degenerative debris in the distal nerve portion of all injured groups and proximal nerve portion of the sedentary diabetic injured group. Dd; degenerative debris. Toluidine blue stained. Bar = 20 μm.

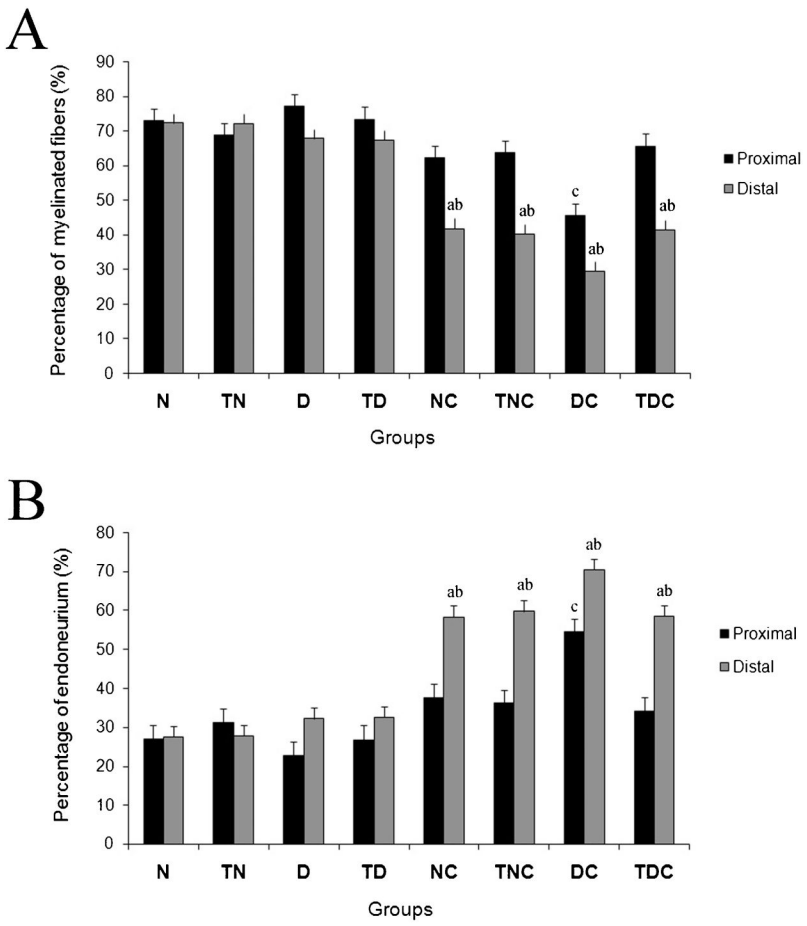

Figure 4 - Graphs showing the morphometrical parameters in the proximal and distal portions of the sciatic nerve in non-diabetic (N, n = 6), trained non-diabetic (TN, n = 6), diabetic (D, n = 6), trained diabetic (TD, n = 8), non-diabetic submitted to sciatic nerve crush (NC, n = 6), trained non-diabetic submitted to sciatic nerve crush (TNC, n = 6), diabetic submitted to sciatic nerve crush (DC, n = 9) and trained diabetic submitted to sciatic nerve crush (TDC, n = 7) groups. A: Percentage of endoneurium and B: percentage of myelinated fibers. Data are expressed as means ± SEM. a $P<0.05$ *vs* proximal portion in the same group; b $P<0.05$ *vs.* distal portion in the N, TN, D, TD groups; c $P<0.05$ *vs.* proximal portion in the N, TN, D, TD, NC, TNC and TDC groups.

TN, D and TD groups) revealed no identifiable alteration to the normal histologic features of sciatic nerve (Fig. 3).

The distal portion of the sciatic nerve of injured rats (NC, TNC, DC, TDC groups) showed a pattern of incomplete histologic regeneration with a predominance of small, thin myelinated fibers, enlargement of the space occupied by endoneurial tissue and the presence of degenerative debris. The proximal portion of the sciatic nerve of the TNC and TDC groups showed normal histological features. However, the proximal portion of the NC and DC groups showed a pattern of incomplete histologic regeneration and, in the DC group, this pattern was similar to that seen in the distal portion (Fig. 3).

Morphometric Studies

In the sciatic nerve of the uninjured rats (N, NT, D and DT), approximately 28% of the area was occupied by endoneurium and around 72% by myelinated fibers (Fig. 4). Furthermore, in the distal portion of the sciatic nerve crush of the NC, TNC, DC, TDC groups, the percentage occupied by endoneurium was higher (~58%, 60%, 70% and 59%, respectively) and the percentage occupied by myelinated fibers was lower (~41%, 40%, 29% and 42%, respectively) than in the uninjured groups and to their values in the proximal portion. Also, in the DC group, the proximal portion of the crushed sciatic nerve showed an increase in the percentage of endoneurium (54.5 ± 3%; $P<0.01$) and a decrease in the percentage of myelinated fibers (45.5 ± 3%; $P<0.01$) when compared to all the other groups (Fig. 4).

Uninjured diabetic groups (D and TD) showed a decrease in the axonal diameter of the sciatic nerve ($P<0.05$; Fig. 5a), while sedentary injured groups (NC and DC) showed a decrease in the myelinated fiber diameter and myelin sheath thickness of the proximal portion of the crushed nerve when compared to uninjured groups ($P<0.05$; Fig. 5b, c). In addition, the DC group had a smaller axonal diameter than the uninjured rats ($P<0.05$; Fig. 5a).

At the distal portion of the crushed nerve, the axonal diameter, the myelinated fiber diameter and the myelin sheath thickness of all the injured groups (NC, TNC, DC, TDC) were smaller than in the uninjured groups (N, TN, D, TD; $P<0.05$; Fig. 5).

Comparing both nerve segments in the injured groups, the distal portion showed an increase in the percentage of endoneurium, a decrease in the percentage of myelinated

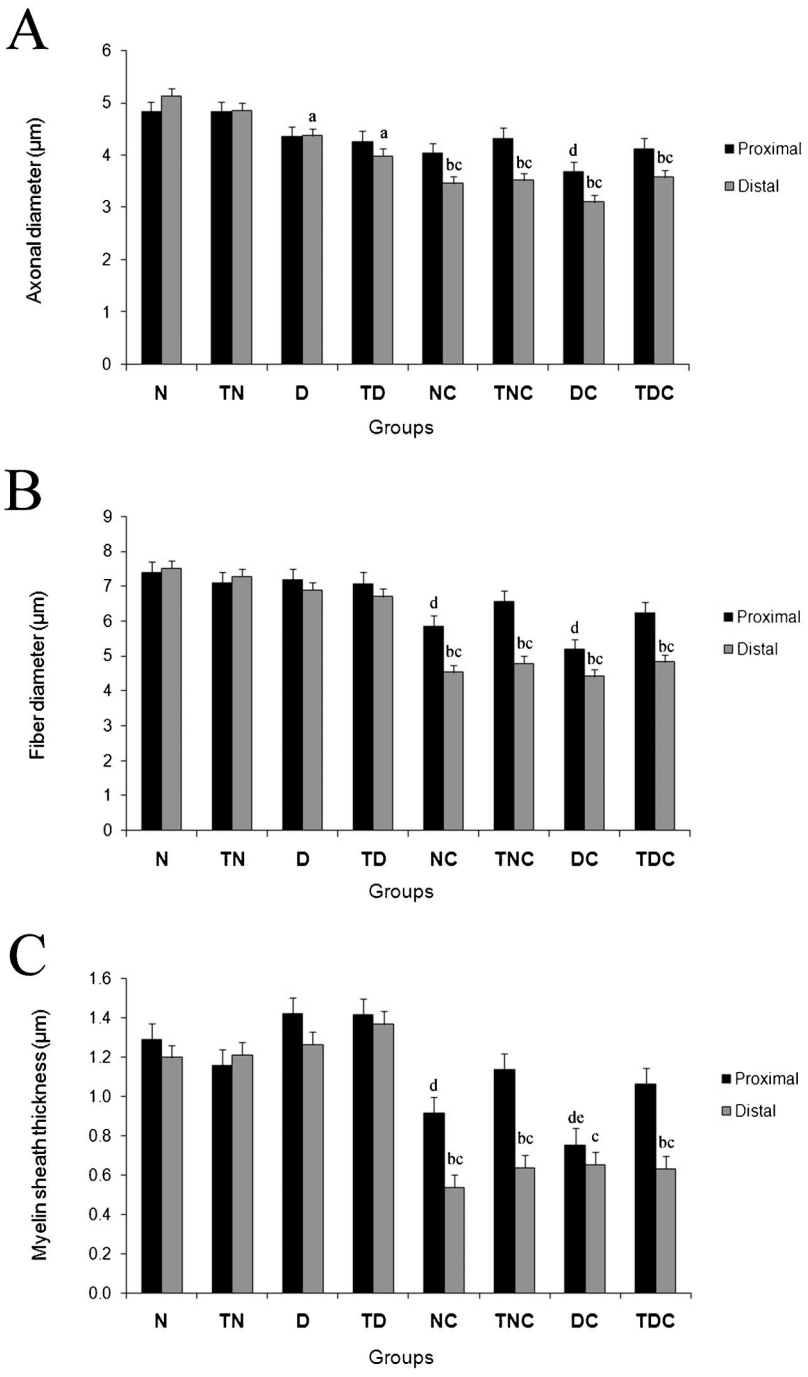

Figure 5 - Graphs showing the morphometrical parameters in the proximal and distal portions of the sciatic nerve in non-diabetic (N, n = 6), trained non-diabetic (TN, n = 6), diabetic (D, n = 6), trained diabetic (TD, n = 8), non-diabetic submitted to sciatic nerve crush (NC, n = 6), trained non-diabetic submitted to sciatic nerve crush (TNC, n = 6), diabetic submitted to sciatic nerve crush (DC, n = 9) and trained diabetic submitted to sciatic nerve crush (TDC, n = 7) groups. A: average axonal diameter; B: average myelinated fiber diameter; C: average myelin sheath thickness. Data are expressed as means ± SEM. a $P<0.05$ *vs.* distal portion in the N and TN groups; b $P<0.05$ *vs.* proximal portion in the same group; c $P<0.05$ *vs.* distal portion in the N, TN, D, TD groups; d $P<0.05$ *vs.* proximal portion in the N, TN, D, TD groups; e $P<0.05$ *vs.* proximal portion in the TNC and TDC groups.

fibers and a decrease in axonal diameter, myelinated fiber diameter and myelin sheath thickness ($P<0.05$). However, there was no difference in the myelin sheath thickness between the proximal and distal portions of the sciatic nerve in the DC group ($P>0.05$; Figs 4 and 5).

In these analyzed morphometric measurements, there were no differences between the N vs TN, D vs TD and NC vs TNC groups ($P>0.05$), and there were also no differences in the densities of the myelinated fibers amongst all the studied groups (data not shown; $P>0.05$).

DISCUSSION

Axonotmesis, commonly seen in crush injury, causes severe sensorimotor impairments and functional disabilities.[16] As seen in our study, crush injury induced a temporary, but complete, loss of function that recovered to control levels by 4 weeks, in non-diabetic rats.[15,17] Complete recovery after crush injury has been explained by the guidance of regenerating axons through their original basal lamina tubes.[18] However, a previous study showed that only 71.4% of the peroneal motoneurons were correctly directed 2 months after sciatic crush injury.[19] The authors suggested that this misdirection may have been caused by damage to the basal lamina tubes by the applied crush technique as seen in others studies.[20]

After sciatic nerve crush, there is a daily increase in SFI values, showing gradual improvement of hindlimb motor function.[15] In our study, in the first three weeks after sciatic nerve crush, injured diabetic rats showed significantly lower SFI values when compared with injured non-diabetic rats. Moreover, while the non-diabetic rats showed signs of functional recovery from post-injury week 4, diabetic rats recovered their motor function only after post-injury week 9. These findings indicate that the spontaneous functional motor recovery is slower in the presence of persistent hyperglycemia and could be because of the defects in nerve regeneration after injury.

Previous studies have shown that nerve regeneration after injury is impaired in experimental[21] and human[22] diabetes as a result of delay in wallerian degeneration time course,[23] elongation rate of axonal sprouts[24] and, subsequently, in nerve fiber maturation.[4]

Although all injured rats displayed motor function recovery at 13 weeks after sciatic nerve crush, evidence of morphologic alterations to the sciatic nerve remained in the proximal and distal nerve portions. In the proximal portion, the injured groups (NC and DC) showed reduction of the myelinated fiber diameter and myelin sheath thickness. The DC group also showed axonal atrophy, a decrease in the percentage of the area occupied by myelinated fibers and an increase in the percentage of the area occupied by endoneurium.

Proximal to the lesion, generally the degeneration stops at the first internode in mild injuries, but may extend further, proximally, in severe injuries.[25] Little is known about the effects of crush on the proximal sciatic portion as most studies focus on the distal portion. A recent study from our laboratory showed a decrease in myelinated fiber and axonal diameter in non-diabetic rats after 7 weeks following sciatic nerve crush.[8] We believe that the findings in the proximal nerve portion of the NC and DC groups may indicate incomplete fiber maturation after retrograde degeneration following sciatic nerve crush and/or by the degeneration process of misdirected axons, which fail to reach the end-organ. Also, a reduced axon diameter represents a characteristic common in diabetes caused by persistent hyperglycemia. In fact, axonal atrophy is a common finding in diabetic peripheral nerves after short- (15 days)[26] and long-term (12 weeks and 12 months) diabetes.[2,27]

Distal to crush injury, in all the groups studied, there were reductions in the myelinated fiber diameter, axonal diameter, myelin sheath thickness and the area occupied by myelinated fiber, and an increase in the area occupied by endoneurium. These morphometric alterations could indicate incomplete spontaneous regeneration after wallerian degeneration of the distal nerve portion. These morphologic alterations in the distal portion of the sciatic nerve were also observed at 3,[17] 7[8] and 12 weeks[15,17] after crush injury in non-diabetic rats, and at 5 and 24 weeks after nerve injury in diabetic rats.[28]

Considering the debility of motor function presented by injured diabetic animals in the first weeks after sciatic nerve crush, in the present study, we chose to start the training protocol in 4th week after the crush injury in order to prevent the deleterious effects of exercise on denervated muscle in the diabetic groups.[29]

In our study, the treadmill training was not able to promote morphometric modifications of the distal nerve portion of the injured nerve. However, the treadmill training accelerated functional motor recovery to the 7th week after sciatic nerve crush of the injured diabetic rats and prevented or reverted the morphologic alterations found in the proximal portion of non-diabetic and diabetic injured sciatic nerve.

Treadmill training after nerve injury of non-diabetic rats produces a marked enhancement of motor axon regeneration[7,30] and enhances the return of sensorimotor function[30,31] without an increase in the proportion of misdirected motor axons to functionally inappropriate targets.[6]

A previous study showed that 7 weeks following sciatic nerve crush the distal nerve portion of the endurance-trained non-diabetic rats (by 5 weeks) showed an increase in myelin sheath thickness and in the percentage of the area occupied by myelinated fibers in comparison with sedentary non-diabetic rats. This study also showed that abnormal morphologic alterations of the proximal portion of sciatic nerve after crush in non-diabetic rats were also prevented or reverted by exercise.[8]

We hypothesized that increasing motoneuron inputs via the spinal circuits that drive locomotion during the regeneration period may influence the functional and morphologic outcome by enhancing fiber maturation which is impaired by diabetes[4] and by crush.[8] Treadmill training may promote the enhancement of fiber regeneration after sciatic nerve crush by potentiating Schwann cell proliferation mediated by phospho-ERK1/2 protein levels[32] and by up-regulation of neurotrophins resulting from increased neuronal activity.[33] In fact, the expression of neurotrophic factors, such as brain-derived neurotrophic factor and neurotrophin 3 were decreased in diabetes[34] and increased by exercise training in muscle and spinal cord.[35]

CONCLUSIONS

Our findings indicate that diabetic condition caused deleterious effects on sciatic nerve regeneration demonstrated by a delay in hindlimb motor function recovery and worse morphometric alterations in proximal nerve portions. In addition, even in the presence of persistent hyperglycemia, a 10-week treadmill training protocol was able to accelerate hindlimb motor function recovery in injured diabetic rats and prevent or revert morphometric alterations in proximal nerve portions in non-diabetic and diabetic injured rats.

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REFERENCES

1. Dyck PJ, Giannini C. Pathologic alterations in the diabetic neuropathies of humans: a review. J Neuropathol Exp Neurol 1996;55:1181-93, doi: 10.1097/00005072-199612000-00001.
2. Filho OAQR, Fazan VPS. Streptozotocin induced diabetes as a model of phrenic nerve neuropathy in rats. J Neurosci Methods 2006;151:131-8, doi: 10.1016/j.jneumeth.2005.06.024.
3. Sima AA, Sugimoto K. Experimental diabetic neuropathy: an update. Diabetologia. 1999;42:773-88, doi: 10.1007/s001250051227.
4. Yasuda H, Terada M, Maeda K, Kogawa S, Sanada M, Haneda M. Diabetic neuropathy and nerve regeneration. Prog Neurobiol. 2003;69:229-85, doi: 10.1016/S0301-0082(03)00034-0.
5. Balducci S, Iacobellis G, Parisi L, Di Biase N, Calandrielo E, Leonetti F, et al. Exercise training can modify the natural history of diabetic peripheral neuropathy. J Diabetes Complications. 2006;20:216-23, doi: 10.1016/j.jdiacomp.2005.07.005.
6. English AW, Cucoranu D, Mulligan A, Sabatier M. Treadmill training enhances axon regeneration in injured mouse peripheral nerves without increased loss of topographic specificity. J Comp Neurol. 2009;517:245-55, doi: 10.1002/cne.22149.
7. Sabatier MJ, Redmon N, Schwartz G, English AW. Treadmill training promotes axon regeneration in injured peripheral nerves. Exp Neurol. 2008; 211:489-93, doi: 10.1016/j.expneurol.2008.02.013.
8. Ilha J, Araújo RT, Malysz T, Hermel EES, Rigon P, Xavier LL, et al. Endurance and resistance exercise training programs elicit specific effects on sciatic nerve regeneration after experimental traumatic lesion in rats. Neurorehabil Neural Repair. 2008;22:355-66.
9. Do Nascimento PS, Malysz T, Ilha J, Araujo RT, Hermel EES, Kalil-Gaspar PI, et al. Treadmill training increases the size of A cells from the L5 dorsal root ganglia in diabetic rats. Histol Histopath. 2010;25:719-32.
10. De Angelis KLD, Oliveira AR, Dall'ago P, Peixoto LRA, Gadonski G, Fernandes TG, et al. Effects of exercise training in autonomic and myocardial dysfunction in streptozotocin-diabetic rats. Braz J Med Biol Res. 2000;33: 635-41.
11. Souza SB, Flues K, Paulini J, Mostarda C, Rodrigues B, Souza LE, et al. Role of exercise training in cardiovascular autonomic dysfunction and mortality in diabetic ovariectomized rats. Hypertension. 2007;30:786-91, doi: 10.1161/HYPERTENSIONAHA.107.095000.
12. Selagzi H, Buyukakilli B, Cimen B, Yilmaz N, Erdogan S. Protective and therapeutic effects of swimming exercise training on diabetic peripheral neuropathy of streptozotocin-induced diabetic rats. J Endocrinol Invest. 2008;31:971-8.
13. Rodrigues B, Figueroa DM, Mostarda CT, Heeren MV, Irigoyen MC, De Angelis K. Maximal exercise test is a useful method for physical capacity and oxygen consumption determination in streptozotocin-diabetic rats. Cardiovasc Diabetol. 2007;6:38, doi: 10.1186/1475-2840-6-38.
14. Varejão ASP, Meek MF, Ferreira AJA, Patrício JAB, Cabrita MAS. Functional evaluation of peripheral nerve regeneration in rat: walking track analysis. J Neurosci Methods. 2001;108:1-9, doi: 10.1016/S0165-0270(01)00378-8.
15. De Medinaceli L. Interpreting nerve morphometry data after experimental traumatic lesions. J Neurosci Methods. 1995;58:29-37, doi: 10.1016/0165-0270(94)00156-B.
16. Navarro X, Vivó M, Valero-Cabré A. Neural plasticity after peripheral nerve injury and regeneration. Prog Neurobiol. 2007;82:163-201, doi: 10.1016/j.pneurobio.2007.06.005.
17. Hoogeveen JF, Troost D, Wondergem J, Van der Kracht AH, Haveman J. Hyperthermic injury versus crush injury in the rat sciatic nerve: a comparative functional histopathological and morphometrical study. J Neurol Sci. 1992;108:55-64, doi: 10.1016/0022-510X(92)90188-Q.
18. Nguyen QT, Sanes JR, Lichtman JW. Pre-existing pathways promote precise projection patterns. Nat Neurosci. 2002;5:861–7, doi: 10.1038/nn905.
19. De Ruiter GC, Malessy MJ, Alaid AO, Spinner RJ, Engelstad JK, Sorenson EJ, et al. Misdirection of regenerating motor axons after nerve injury and repair in the rat sciatic nerve model. Exp Neurol. 2008;211:339-50, doi: 10.1016/j.expneurol.2007.12.023.
20. Bodine-Fowler SC, Meyer RS, Moskovitz A, Abrams R, Botte MJ. Inaccurate projection of rat soleus motoneurons: a comparison of nerve repair techniques. Muscle Nerve. 1997;20:29–37, doi: 10.1002/(SICI)1097-4598(199701)20:1<29::AID-MUS4>3.0.CO;2-J.
21. Kennedy JM, Zochodne DW. The regenerative deficit of peripheral nerves in experimental diabetes: its extent timing and possible mechanisms. Brain. 2000;123:2118–29, doi: 10.1093/brain/123.10.2118.
22. Bradley JL, Thomas PK, King RH, Muddle JR, Ward JD, Tesfaye S, et al. Myelinated nerve fibre regeneration in diabetic sensory polyneuropathy: correlation with type of diabetes. Acta Neuropathol. 1995;90:403–10, doi: 10.1007/BF00315014.
23. Terada M, Yasuda H, Kikkawa R. Delayed Wallerian degeneration and increased neurofilament phosphorylation in sciatic nerve of rats with streptozotocin-induced diabetes. J Neurol Sci. 1998;155:23-30, doi: 10.1016/S0022-510X(97)00269-4.
24. Ekström PAR, Kanje M, Skottner A. Nerve regeneration and serum levels of insulin-like growth factor-1 in rats with streptozotocin-induced insulin deficiency. Brain Res. 1989;496:141-8, doi: 10.1016/0006-8993(89)91060-3.
25. Campbell WW. Evaluation and management of peripheral nerve injury. Clin Neurophysiol. 2008;119:1951-65, doi: 10.1016/j.clinph.2008.03.018.
26. Salgado HC, Fazan Júnior R, Fazan VP, Da Silva VJ, Barreira AA. Arterial baroreceptors and experimental diabetes. Ann N Y Acad Sci. 2001;940:20-7, doi: 10.1111/j.1749-6632.2001.tb03663.x.
27. Bestetti G, Rossi GL, Zemp C. Changes in peripheral nerves of rats four months after induction of streptozotocin diabetes. A qualitative and quantitative study. Acta Neuropathol. 1981;54:129-34, doi: 10.1007/BF00689405.
28. Terada M, Yasuda H, Kikkawa R, Shigeta Y. Tolrestat improves nerve regeneration after crush injury in streptozotocin-induced diabetic rats. Metabolism. 1996;45:1189-95, doi: 10.1016/S0026-0495(96)90234-6.
29. Herbison GJ, Jaweed MM, Diturnno JF, Scott CM. Effect of overwork during reinnervation of rat muscle. Exp Neurol. 1973;41:1-14, doi: 10.1016/0014-4886(73)90176-3.
30. Marqueste T, Alliez J-R, Alluin O, Jammes Y, Decherchi P. Neuromuscular rehabilitation by treadmill running or electrical stimulation after peripheral nerve injury and repair. J Appl Physiol. 2004;96:1988–995, doi: 10.1152/japplphysiol.00775.2003.
31. Seo TB, Han IS, Yoon JH, Hong KE, Yoon SJ, Namgung U. Involvement of Cdc2 in axonal regeneration enhanced by exercise training in rats. Med Sci Sports Exerc. 2006;38:1267-76, doi: 10.1249/01.mss.0000227311.00976.68.
32. Seo TB, Oh MJ, You BG, Kwon KB, Chang IA, Yoon JH, et al. ERK1/2-mediated Schwann cell proliferation in the regenerating sciatic nerve by treadmill training. J Neurotrauma. 2009;26:1733-44, doi: 10.1089/neu.2008.0711.
33. Chan JR, Cosgaya JM, Wu YJ, Shooter EM. Neurotrophins are key mediators of the myelination program in the peripheral nervous system. Proc Natl Acad Sci USA. 2001;98:14661-8, doi: 10.1073/pnas.251543398.
34. Rodríguez-Peña A, Botana M, González M, Requejo F. Expression of neurotrophins and their receptors in sciatic nerve of experimentally diabetic rats. Neurosci Lett. 1995;200:37-40, doi: 10.1016/0304-3940(95)12067-E.
35. Gómez-Pinilla F, Ying Z, Opazo P, Roy RR, Edgerton VR. Differential regulation by exercise of BDNF and NT-3 in rat spinal cord and skeletal muscle. Eur J Neurosci.