Morphological and functional aspects of sciatic nerve regeneration after crush injury

ANDREEA RĂDUCAN1)*, SILVIA MIRICĂ1)*, OANA DUICU1), S. RĂDUCAN2), DANINA MUNTEAN1), O. FIRA-MLĂDINESCU1), RODICA LIGHEZAN3)

1)Department of Pathophysiology
2)Department of Human Anatomy and Embryology
3)Department of Histology
"Victor Babeş" University of Medicine and Pharmacy, Timişoara

*The authors have contributed equally to this work.

Abstract
Experimental models for the investigation of nerve regeneration are critical in studying new strategies able to promote the repair process. The aim of the present work was to characterize morphological and functional aspects of sciatic nerve regeneration after mechanical crush injury in rodents. Morphological changes were assessed after a four minutes sciatic nerve injury induced by means of a standardized compression clip. Rat nerve samples were collected before injury and after 24 hours, four days, two weeks, and four weeks after injury, respectively. In an additional group with unilateral sciatic nerve injury, animals were evaluated for four weeks using walking track analysis and the sciatic static index (SSI) measured in both rearing and normal standing position. Histological study showed important axonal degeneration at four days and axonal regeneration at four weeks after injury. We observed no significant differences between SSI in rearing and normal standing stance and a strong correlation between SSI values measured in the two positions during the evaluation period. Positive correlations were also found for the footprint parameters. Our data provide a baseline characterization of the sciatic nerve crush injury that will further allow the investigation of peripheral nerve regeneration in the presence of potential neuroprotective agents in post-traumatic nerve repair.

Keywords: crush injury, nerve regeneration, functional recovery, axonal morphology.

Introduction
During the last decades, important advances have been made in the field of posttraumatic peripheral nerve regeneration [1–7] with respect to the repairing strategies of peripheral nerves [8–10]. This is particularly important since in clinical practice nerve injuries are much more frequent than spinal cord injuries [11, 12]. Although not always life threatening, the first ones do present a considerable socio-economical impact.

The rat sciatic nerve model of crush injury is widely used to assess the post-traumatic impairment of motor function [13–16] offering some advantages over the nerve transaction model [13, 17–19].

The aim of the present study was to assess the morphological and functional characteristics of sciatic nerve regeneration after a previously standardized crush injury [20] as control for further studies of nerve regeneration in the presence of neuroprotective agents.

Materials and Methods
All experimental procedures were conducted according with the Directive 2010/63/EU on the protection of animals used for scientific purposes. The experimental protocol was approved by the Ethics Committee of the “Victor Babeş” University of Medicine and Pharmacy, Timişoara, Romania. Animals were fed ad libitum and housed under standard conditions (constant temperature and humidity of 22.5±2°C and 55±5%, 12 hours light/dark cycle).

Twenty-seven Sprague-Dawley female rats, weighing 250–300 g were used. All surgical procedures were performed under deep anesthesia using Ketamine (70 mg/body weight) and Xylazine (10 mg/body weight) i.p. Animals were placed in prone position. After asepsia and incision of the skin, the right sciatic nerve was exposed through a biceps muscle splitting incision in the posterior thigh. A four minutes long crush injury was performed 10 mm proximal to the bifurcation of the sciatic nerve using a custom-made compression clip (Figure 1, A and B). The technique has been previously standardized to develop a constant compression force of 98.182±0.22 cN (coefficient of variation = 0.61%) [20]. Compression injury was electrophysiologically monitored using two pairs of electrodes to record the compound action potential before injury and to assess its progressive decrease to 0 mV during injury. Afterwards, the muscle and skin were closed by layers using 5/0 absorbable sutures (Ethicon Vicryl®).

In order to assess morphological changes, nerve samples distal to the crush injury were collected at 24 hours (n=3), four days (n=3), two weeks (n=3) and four weeks (n=3) after injury, respectively. Nerve samples (n=3) from a corresponding level were also withdrawn from animals without crush injury to be used as control. Animals were then euthanatized.
Nerve samples were then fixed and prepared for the morphological assessment according to the following protocol: fixation by immersion in glutaraldehyde (24 hours), post-fixation in 1% osmium tetroxide (60 minutes), washing in phosphate buffer (2×10 minutes), dehydration with ascending (70%, 80%, 96%, and 100%) ethanol passages (4×20 minutes for each solution), and infiltration with propylene oxide. Specimens were then embedded in resin (polymerization at 60°C, 48 hours). Finally, series of 1-μm thick semi-thin transverse sections were cut starting from the distal stump of the sciatic nerve sample and stained with Richardson’s staining (1% Methylene Blue prepared in 1% Borax, mixed in equal parts with 1% Azure II). The stained semi-thin sections were analyzed by means of a light microscope Nikon E600 equipped with a Coolpix 950 digital camera (Nikon Corporation Co., Ltd., Japan) and the following parameters were estimated: total fibers’ number and density, diameter of axons and nerve fibers, and myelin thickness.

In a separate group of animals (n=12), unilateral sciatic nerve injury was performed and assessed daily for four weeks post-injury by means of footprint analysis. We used the same protocol of crush injury as described above for the morphological assessment. Footprint collection was made by a video imaging technique for the injured (right) and the uninjured (left) hind limb and the following parameters were measured: (i) print length – PL, the distance between the tip of the third toe and the most posterior part of the foot; (ii) 1–5 toe spread – TS, the distance between the first and the fifth toes; (iii) 2–4 (intermediary) toe spread – ITS, the distance between the second and the fourth toes. Sciatic static index (SSI) was calculated in both rearing (SSIm2) and normal standing position (SSIm4) according to the formula [21]:

\[
SSI = 108.44 \times \frac{TSe - TSn}{TSn} + 31.85 \times \frac{ITSe - ITSn}{ITSn} - 5.49
\]

n – normal, parameter for the uninjured hind limb;

\[c\] – experimental, parameter for the injured hind limb.

All pictures of the rat hind feet were acquired with a digital camera (Olympus Camedia C-3040 Zoom). After a short accommodation period, five pictures were taken for each animal in normal standing stance (supported by all four paws) and five pictures in rearing stance (supported by the hind limbs only), respectively. Measurements of the parameters was performed using an image processing and analysis program (Image J 1.38x).

Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, USA) Statistical significance was established as \(p<0.05\).

**Results**

The morphological study of qualitative alterations in crushed sciatic nerves revealed at 24 hours a relative normal aspect (Figure 2).

The most evident changes caused by axonal degeneration could be observed after four days post-injury and consisted in axonal swelling and disintegration of axonal cytoskeleton elements, associated with myelin degradation in the presence of an intense phagocytic process (Figure 3).

At four weeks after crush injury, regenerated nerve fibers were present characterized by a smaller size and a thinner myelin sheath in comparison to controls. They were organized in small fascicles (Figure 4). Some degeneration features could still be observed among the many regenerated nerve fibers. The presence of these fibers in an early phase of myelinization indicate that at one month post-injury the regeneration process is still incomplete, the myelinization process being unfinished at this point.

After four weeks, the number and density of the regenerated myelinated axons was higher compared to controls.

Functional results of the footprint analysis throughout the four-week post-operative period are depicted in Figure 5.

The applied standardized crush injury was responsible for a significant functional loss as assessed by SSI. The functional loss was considered zero before crush injury, meaning no functional loss was present. Negative values indicate the degree of functional loss (maximum – 100 post-crush). Statistical analysis on SSI values revealed significant differences between pre-operative condition and the first three weeks after crush lesion (\(p<0.05\)) for both evaluated positions.
We observed a marked decrease on function in the first week, followed by a gradual recovery of normal gait during the following weeks, so that by the end of the fourth week animals regained normal gait as assessed by SSIm2 and SSIm4 ($p>0.05$ for SSI preoperative vs. SSI at four weeks).

Statistical analysis performed with one-way ANOVA and Tukey–Kramer multiple comparisons method showed during the 28 post-operative days no significant difference for SSI between images obtained in normal stance and those obtained in rearing stance ($p>0.05$).

Using Pearson’s index, we established correlations between footprint parameters collected in normal standing and in rearing stance for both the injured and the uninjured hind limb. Our results indicate that there are statistical significant positive correlations in the case of all recorded footprint parameters. Accordingly, there was a strong correlation for injured TS ($r=0.89$) and injured ITS ($r=0.77$), respectively. We also found a significant correlation between SSIm2 and SSIm4 ($r=0.99, p<0.0001$) (Figure 6).

Low correlations were present for injured PL ($r=0.29$) and uninjured PL ($r=0.34$). The analysis of the parameter’s reproducibility showed coefficients of variations ranging from 7.73% (TS, uninjured hind limb, rearing stance) to 26.52% (PL, injured hind limb, rearing stance). Coefficients of variations were always higher when measured in the injured hind limb compared to the uninjured side for both assessment positions. Measurement of TS appeared to be the most accurate, with coefficients of variations ranging from 7.73% to 10.67%.

Figure 2 – Transverse section of a normal sciatic nerve, semi-thin section (1 μm). Richardson’s staining, ×200.

Figure 3 – Wallerian degeneration of an injured sciatic nerve; numerous mast cells in the endoneurium, semi-thin section (1 μm). Richardson’s staining, ×400.

Figure 4 – Remielinisation. Cross section of the sciatic nerve: thick myelinated nerve fibers together with small groups of nervous fibers with thin myelin sheet; semi-thin section (1 μm). Richardson’s staining, ×400.

Figure 5 – Comparison of the functional recovery assessed by SSI in rearing and normal standing position.

Figure 6 – Correlation between SSI in rearing and normal standing position.
Discussion

The crush injury (axonotmesis) of the sciatic nerve in rats is a widely used experimental model in nerve regeneration studies of the peripheral nervous system and various methods have been described in the literature to perform this type of injury, including several compression devices and various crush durations [13, 17, 18, 22]. Also, in the last years some study groups started to use the forelimb as an alternative to hindlimb nerve models of nerve regeneration research [23, 24]. However, the sciatic nerve model remains the most used and reliable experimental approach in nerve regeneration studies due to the several behavioral functional tests available such as the computerized gait analysis [13, 16, 18].

In our study, after the standardized crush injury was applied, a complete functional loss was noticed in all rats. Electrophysiological assessment of nerve conduction ability was performed in order to ensure standardization of the crush injury and to confirm the nerve injury by recording loss of compound action potential after compression. We obtained a full recovery after 28 days post-injury as indicated by the SSI, but according to the literature there are considerable differences between the recovery periods reported by other researchers, usually between three and eight weeks for models of axonotmesis [25–28]. This is most likely due to the different compression devices that were used in order to cause the sciatic nerve lesion. The experiment was ended up at four weeks post-operative since at this stage animals reached normal functional test performance objectified by values of the SSI comparable to preoperative measurements in both positions. During our study, carefully animal surveillance was provided, especially during the early post-operative period. No autotomy, skin ulcers, infections or joint contractures were noticed at variance from data reported by other groups during the post-operative period [21, 29].

Our data are in line with the results reported by Bozkurt A et al. [16] supporting the fact that SSI can be measured in both rearing and normal stance without significant differences regarding nerve regeneration outcome, although these authors used the model of rat sciatic nerve transaction. Since the calculation of the SSI requires only two footprint parameters (TS and ITS), for a complex functional evaluation in rearing and normal position, we decided to study also PL. Our results show that, when comparing the two positions, the measurement of PL presents the lowest correlations, whereas the measurement of TS seems to be the most reliable.

The study of myelinated axons is probably the most important morphological feature that provides data about pathophysiology of crush injury [30]. Since osmium tetroxide, used for nerve staining, is very toxic and not currently used by many histologists, safety procedures for osmium tetroxide handling, storage and removal were respected. Our data confirmed that nerve fiber regeneration and myelinisation evolve faster in crush injury models as compared to nerve transection models due to the preservation of continuity of the epineurium. At four weeks after injury, the presence of small nerve fibers surrounded by thin myelin sheaths indicated the presence of nerve regeneration, but it was a yet unfinished process and features of nerve degeneration were still present. Also, we observed differences between the histological parameters in crushed sciatic nerve fibers compared to controls, i.e., the number and density of the regenerated myelinated axons was higher in nerve samples collected from rats with sciatic nerve lesion. However, despite the preservation of continuity of the epineurium a full recovery assessed by both morphological and functional tests was not achieved after complete axonotmetic injury. This observation is in line with other reports in the literature [13, 24].

Conclusions

The in vivo rat experimental model of sciatic nerve crush injury is reproducible and suitable for further investigations regarding the effects of potential neuroprotective agents. The use of both functional and morphological assessment methods is recommended in order to ensure a comprehensive evaluation of nerve regeneration and functional recovery after peripheral nerve lesions. Measurement of SSI as a method to assess functional recovery after crush injury is advantageous since it can be performed independent of the animal position.

References

Morphological and functional aspects of sciatic nerve regeneration after crush injury


Corresponding author
Ovidiu Fira-Mălădinescu, Associate Professor, MD, PhD, Department of Pathophysiology, “Victor Babes” University of Medicine and Pharmacy, 2 Eftimie Murgu Square, 300041 Timișoara, Romania; Phone +40256–493 085, e-mail: mladinescu@umft.ro

Received: March 5, 2013

Accepted: September 21, 2013