Neuroscience

Analysis of Myelin and Neurofilament Content in a Sciatic Nerve Crush Injury Model

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Experimental models exploring the ultrastructural changes that follow peripheral nerve injury are critical in our efforts to better understand the outcomes after such injuries and to advance our current clinical models of peripheral nerve injury. Furthermore, an understanding of the underlying architecture following injury lends insight into the mechanisms that may be at work when pharmacological interventions are introduced. The aim of the present work was to quantitatively measure and evaluate the damage to myelin and neurons following calibrated crush injuries at 3 and 5 days post-injury (DPI) in mouse sciatic nerves at and adjacent to the crush site. Using immunohistochemical methods, we measured myelin and neuron content by staining for myelin protein zero (P0) and neurofilament (NF). This histological analysis revealed severity dependent levels of damage to both myelin and neurofilament. Additionally, the data revealed distinct patterns of injury evolution for myelin and neurons between 3 and 5 DPI. Our data describes the state of two key components of the nerve – myelin and neurons – shortly after injury and also reveals their early patterns of injury evolution. This data will allow for further investigation into the structure of peripheral nerves following injury, and the possible sites of action for therapeutic adjuvants.

Peripheral Nerve Injury | Sciatic Nerve Crush | Myelin | Myelin Protein Zero (P0) | Neurofilament (NF)

Introduction

Peripheral nerve injury is a consequence of trauma to the extremities with unpredictable capacity for recovery. The severity and location of the injury may determine the optimal clinical intervention and prognosis. A mild peripheral nerve injury has the ability to regenerate without invasive treatment, whereas surgical intervention may be required for more severe injuries [1]. It is critical to assess the severity of the injury to determine the optimal course of treatment, however current methods of distinguishing type and severity of injury may delay definitive diagnosis for weeks post-injury [2, 3]. The staging of peripheral nerve injury according to the currently accepted classification lacks the ability to predict outcome. The classification of Seddon and Sunderland applies characteristics of individual neurons to entire nerves, defining injuries descriptively as neuropraxia, axonotmesis, or neurotmesis [4, 5]. However, the reality of peripheral nerve injuries is more complex. In a large, injured peripheral nerve, severed axons exist alongside intact axons and demyelinated axons, with variability in structural integrity and the capacity for functional improvement [6]. The difficulty in effectively classifying peripheral nerve injuries creates a diagnostic dilemma.

Many studies characterize peripheral nerve degeneration and regeneration using a sciatic nerve crush injury model with behavioral, morphological, and electrophysiological techniques used to measure recovery [7-11]. The picture of injury that emerges is a complex mixture of injury to myelin and neurons [7-10]. However, there is no established method of comparing the degree of damage to these two separate components. We have previously shown that pharmaco-therapeutics such as erythropoietin (EPO) can accelerate peripheral nerve healing to rates faster than neuronal regeneration models would suggest possible [6]. We hypothesized that early functional improvement from peripheral nerve injury may be the result of improvements on the cellular ultrastructure of a peripheral nerve after injury at the earliest time points. Therefore, we sought to determine the percentage of the crushed nerve that remains intact to support the functional improvement in the initial days after injury. This was assessed through quantitative immunohistochemistry (IHC) staining for neurofilament (NF), and myelin, two important yet distinct components of the peripheral nervous system. We further hypothesized that this injury would propagate spatially both proximally and distally in the injured nerve, and examined sites proximal and distal to the crush site to evaluate this effect.

Materials and Methods

Surgery

All animal procedures were approved by the University Committee of Animal Resources at our institution. Ten-week-old female C57BL6 mice, weighing 20 to 25 g were anesthetized with intraperitoneal ketamine (60 mg/kg) and xylazine (4mg/kg). The left hind limbs were shaved, washed with alcohol, and prepared with povidone-iodine (betadine). Lateral skin incisions were made to expose the sciatic nerve, and mice were randomly subjected to either moderate or severe crush injuries. Moderate crush injuries were created with calibrated forceps (Miltex 18-1107 Swiss Cilia Forceps; Integra Militex; York, PA) held on the sciatic nerve for 30 seconds. To ensure consistent pressure, the forceps were held down with a calibrated pressure jig [12]. Severe crushes were performed with smooth tipped stainless steel needle holders (Webster needle holders RH2560; V. Mueller, CareFusion Corp., San Diego, CA) closed to the first of three notches. These needle holders were closed on the sciatic nerve for 30 seconds for the severe crushes. This technique of moderate and severe crush injuries generates forces of 4.89 MPa and 9.53 MPa, respectively [12].

Immunohistochemistry

Tissue Preparation

At either 3 or 5 days post injury (DPI), mice were sacrificed and sciatic nerves were harvested to include the crush site as well as the areas immediately proximal and distal to it (2mm proximal and distal to the injury site). After excision, sciatic nerves were rinsed in ice-cold PBS followed by fixation in 4% paraformaldehyde at 4°C for 4 hours. Nerves were then washed with PBS and dehydrated. Dehydration was followed by a warm xylene wash and embedding in paraffin. The nerves were then sectioned at 5 µm and stained for histology.

Immunofluorescence was performed using antibodies to myelin protein zero (P0) (Aves Lab, Tigard, OR; Cat# PZO, 1:1000) and antibodies to neurofilament (NF) (Aves Lab, Tigard, OR; Cat# NFH, 1:1000) to examine myelination status and neuron continuity, respectively (Figure 1). Using fluorescent images captured with Axiovision software (Axiolab; Carl Zeiss), computational analysis was performed with ImageJ (http://imagej.nih.gov) software to quantitate P0 and NF [13]. Using ImageJ, positive staining on fluorescent images were first converted to a binary format, which enabled automation of the counting of positive signals, defined as fluorescent of either myelin or neurofilament. This quantification was verified by manual counts before the procedure was applied to all of the images.

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Figure 1.

*All images are representative Sections at Day 3

Figure 2.
Average signal densities were then calculated using 15 representative areas of 50 x 50 m from each section in a fashion consistent with other density quantification methods [14, 15]. Signal densities were calculated as the average number of positive signals per 2500 m². This method prevented inclusion of damaged, or otherwise compromised areas of the image and controlled for variations in cross-sectional area of the sciatic nerve samples. The same image processing was performed on each image following the particle analysis procedure outlined by the user guide for ImageJ.

**Results**

Sciatic nerves were harvested from control mice to measure the amount of intact myelin and neurofilament in untreated, uninjured nerve (Figure 1 Panel A) on the third DPI. In the control group, largely intact P0 myelin staining (red) and neurofilament (green) is shown. In the setting of a moderate crush, marked destruction is most evident at the site of crush injury with relative sparing proximal and distal to the injury site (Figure 1 Panel B). This pattern of destruction is evident but more pronounced in the severe crush injury with the effects of the crush evident both proximal to and distal to the site of injury itself at this early 3 day time point.

Quantitative analysis was performed on moderately and severely crushed sections of nerve, to measure the time-course of the ultrastructural changes to NF and P0 at 3 and 5 DPI (Figure 2). Injury at the crush site propagates proximally and distally so that all injured nerves show less NF and P0 staining intensity at all positions even at three DPI (p < .05). Also, injury at all sites evolves over time such that more profound decreases in NF and P0 continue over the two days from 3 to 5 DPI. Moreover, in the evolution of this injury over the 48 hours after day 3, the decreases in P0 staining are greater than that of NF.

This final point is made more clear when the data are tabulated in terms of the percentage of fibers remaining intact at each site of injury (Figure 3). By 5 DPI, a moderate crush injury only affects 18% of the neurons proximal to the crush site, but a severe injury destroys over 40% (Figure 3). Notably, the crush site itself exhibits severity dependent destruction of neurons in moderate...
relationship between a crush and the area around it. Much like the itself, then these effects could also explain our findings as the current models [16]. If we suppose that nerve injury at the site of a promote recovery far in advance of the time frame which would be classification in our patients and for a clearer relationship between the injury severity dependent effects of crush injury in general [6].

Our use of this model has allowed us to characterize morphological changes associated with pharmacologic adjuvant treatment of peripheral nerve injury, but we have not fully investigated either the effects of agents on the structural components of the nerve, or the injury severity dependent effects of crush injury in general [6]. Such a characterization seems central to the relationship to injury classification in our patients and for a clearer relationship between injury classification and prognosis [2-5]. The basis for this particular work therefore lies in the fact that some agents seem to promote recovery far in advance of the time frame which would be expected if neurons were regenerating in a manner consistent with current models [16]. If we suppose that nerve injury at the site of a crush also has an effect distal and proximal to the site of crush itself, then these effects could also explain our findings as the relationship between a crush and the area around it. Much like the effects of crush intensity, this relationship is poorly understood and uncharacterized in the literature. Our hypotheses in this work are therefore centered around characterizing the main constituents of the nerve in the days following injury both proximal and distal to the crush site. Specifically, we hypothesized that myelin and neuron injury after crush would evolve over the two days after injury (a time period relevant from our previous work) and propagate proximally and distally from the crush site. We chose to examine myelin content as captured by P0 staining and neuron fiber content as captured by NF staining and hypothesized that increased myelin destruction may serve to better explain the stabilizing effect of erythropoietin in the first week after treatment [6]. We chose to use immunohistochemical methods owing to the difficulty in manipulating nerves so soon after a crush injury and our intent to examine specifically the myelin content.

In evaluating morphological changes shortly after injury, we observed unique patterns of destruction that were distinct for neurons and myelin. On day 3, at the site of a moderate crush, 43% of the neurofilament staining was lost, but much less was lost just millimeters proximal to the site of crush injury (~10%). However, distal to the crush site, the amount of neurofilament staining lost remains mostly unchanged from the crush site (47% vs. 43%). In comparing the destruction to neurons between moderate and severe crushes, we see the same pattern emerge, but with greater levels of destruction for severe crushes. Over 48 hours, between days 3 and 5, there is a relatively uniform increase in damage to neurons at all sites. Taken together, this suggests that there is severity dependent destruction of neurons so that the distal uninjured area has no more neurons available to conduct signals than the crush site itself.

In the moderate crush, we see 36% of myelin destroyed by day 3 at the crush site. Adjacent to the crush site, we see 17% of the myelin destroyed proximally and 26% distally, at this 3 day time point. A severe crush increases myelin destruction at the crush site to 50%. Similar to the destruction seen with NF, we again observe severity dependent destruction at the crush site. In the adjacent sites in the severe crush group, 7% of the myelin was destroyed proximally, and 36% distally. Less severe injuries may preferentially injure myelin over neurons, a finding which deserves further investigation. Examining the changes to myelin over time revealed a very pronounced evolution of injury. In moderately crushed nerves, myelin destruction increases by 10% (proximal), 20% (crush site) and 24% (distal) between days 3 and 5. Similarly impressive changes are seen in the severely crushed nerve with increases of 24%(proximal), 16%(crush site) and 9%(distal) in myelin destruction.

**Conclusion**

Examining the morphological changes of crush injuries has allowed us to speculate as to why agents like EPO may work in a time and injury severity dependent manner. It is possible that agents which stabilize myelin can stabilize the injury to a nerve and preserve function early after injury. The number of neurons remaining intact after an injury may be a significant factor in determining the potential for early recovery.


