Structural analysis of a ligatured rat sciatic nerve in the ex vivo state using synchrotron small-angle X-ray scattering (SAXS)

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Background: To understand the fundamentals of neural tissue injury, experiments on the nano-structured nerve system of animals are essential. This study was designed to reveal the nanostructure changes of an isolated ligatured rat sciatic nerve using the synchrotron small-angle X-ray scattering (SAXS) technique.

Methods: Male Sprague-Dawley rats (weighing approximately 250 grams) were used in this study. The SAXS patterns of 1 week after ligatured nerves (N = 5) and the normal sciatic nerves (N = 5) for the control were acquired after extracted approximately 15 mm before the experiment. Experiments were conducted at the 4C1 beam line at the Pohang Accelerator Laboratory in Korea. The exposure time was 60 sec, and 8 to 12 images per sample were acquired in 0.5 mm intervals, including the regions above, around and below the ligatured position.

Results: The periodic peaks of the myelin sheath and the interfibrillar space of collagen completely disappeared at the ligatured position. Farther from the ligatured point, weak and quite different SAXS patterns were observed for the myelin sheath and interfibrillar space. However, the collagen fiber peaks appeared at all positions, although they were weaker near the ligatured position.

Conclusions: The ligature treatment totally destroyed the myelin sheath and interfibrillar space of collagen. In addition, retrograde degeneration developed 2 mm above the ligatured site. The myelin sheath and interfibrillar space of collagen were damaged 6 mm below the ligatured site. However, the collagen fiber structure was not significantly affected by the ligature, indicating a much different structural organization.

Key Words: Myelin sheath, Nanostructure, Sciatic nerve ligation, Small angle X-ray scattering.

INTRODUCTION

Neuropathic pain has characteristic symptoms, such as spontaneous burning, shock-like pain, allodynia, and hyperalgesia, and it is caused by nerve tissue injury. Small animal experiments and an imaging technique that shows nerve nanostructures are essential for investigating neural tissue injury. Nerve tissue examinations using a conventional light microscope or electron microscope inevitably require complex sample preparations (e.g., fixation, sectioning, and staining), thus necessitating considerable processing time and many tissue slices. Furthermore, chemical preparations may damage the nerve structure [1], and hematoxylin-eosin staining may cause artifacts [2,3].

In contrast, the synchrotron small-angle X-ray scattering (SAXS) technique, which uses high-flux synchrotron radiation, has been widely used for the structural analysis of biomaterials ranging in size from a few nm to several hundred nm, and SAXS can provide structural information about organs in real time without sample preparation [4-7]. Thus, the SAXS technique might be a promising tool for future clinical structural analysis.

In this study, which used the synchrotron SAXS technique, we demonstrated the nanostructural changes of isolated ligatured rat sciatic nerves after treatment for peripheral nerve injury. Those results were interpreted and compared with previous histologic observations and analysis of nerve tissue.
MATERIALS AND METHODS

The study protocol was approved by the Institutional Review Board (IRB, 2010-1222-CU-AEC-8-Y). The laboratory animals were treated according to the guidelines for the care and use of laboratory animals, as described by Institutional Animal Care and Use Committee (IACUC) of our hospital.

Experiments were performed using male Sprague-Dawley rats \( (N = 10) \) weighing approximately 250 grams. All animals were kept in a temperature-controlled room with a 12 h light-dark cycle and were acclimated for at least 7 days before use. The animals had free access to water and a standard laboratory diet. All surgery was performed under anesthesia with ketamine (80 mg/ml), which was injected intraperitoneally. Adequate anesthesia was regarded as no movement in response to painful stimulation in the tail. If there was any movement during the surgery, additional dose of ketamine was injected into the peritoneal cavity.

For the experiments \( (N = 5) \), a deep incision was made through the gluteal muscles to find a sciatic nerve in the rat in the prone position. The mid-point of the exposed sciatic nerve was tightly ligatured by 4.0 black silk to induce mechanical damage. When the procedure was completed, the wound was closed with silk layer by layer. The rats were allowed to recover in their normal environment. One week after the surgery, a fragment of the sciatic nerve \( (N = 5) \) covering the ligation point was removed just before the SAXS measurements. For comparison, a normal sciatic nerve \( (N = 5) \) was extracted approximately 15 mm just before the SAXS measurements. The nerve fragment (approximately 15 mm) was sealed between polyamide films to protect from the deformation caused by water evaporation.

The SAXS measurements were conducted at the Pohang Light Source (PLS) 4C1 SAXS 1 beamline of the Pohang Accelerator Laboratory in Korea. The X-rays from the bending magnet were monochromated at 11 keV using a double multilayer monochromator and focused at the detector position using a toroidal mirror. The SAXS patterns were recorded with a two dimensional CCD (charged couple device) detector (MarCCD, Mar USA, Inc. Evanston, IL, USA) [6], and the X-ray irradiation time was 60 seconds. The scattering angles were calibrated using silver behenate \( (\text{AgC}_2\text{H}_4\text{O}_2, d_{001} = 58.380 \, \text{Å}) \), and the sample-to-detector distance was approximately 3 m. The SAXS patterns were measured at 8-12 different spots separated by 0.5 mm.

RESULTS

Normal rat sciatic nerve

The two dimensional (2D) SAXS image and one dimensional (1D) SAXS profiles of the normal sciatic nerve are shown in Fig. 1, where a nerve sample was located parallel to the meridional direction. Quite different SAXS patterns were found along the equatorial and meridional directions (Fig. 1A), and this difference is clearer in the 1D SAXS profiles (Figs. 1B and C). The myelin sheath showed a pattern with a period of 18 nm \( (\text{Fig. 1B, M }_{1\text{st}}, \text{M }_{2\text{nd}}, \text{and M }_{3\text{rd}}) \) in the horizontal direction. The measured interfibrillar distance of collagen was 67 nm; a periodic peak pattern \( (\text{Fig. 1B, F }_{1\text{st}}, \text{F }_{2\text{nd}}) \), which was wide and weak, appeared in the horizontal profile. The collagen showed a 67 nm periodic pattern in the vertical direction \( (\text{Fig. 1, C }_{1\text{st}}, \text{C }_{2\text{nd}}, \text{C }_{3\text{rd}}, \text{C }_{4\text{th}}, \text{C }_{5\text{th}}\text{, and C }_{6\text{th}}) \). The odd-numbered peaks had large widths and high intensities.

Ligatured rat sciatic nerve

The 2D SAXS images and 1D SAXS profiles measured at the normal, ligation point, 2 mm above and 6 mm below the ligation site are shown in Figs. 2 and 3. In the horizontal direction, the periodic patterns of the myelin sheath and the interfibrillar space of collagen were not visible (Figs. 2 and 3). At the points 2 mm above and 6 mm below the ligation site, weak and quite different SAXS patterns were observed for the myelin sheath and interfibrillar space compared with those of the normal nerve (Figs. 2 and 3). The positions of the collagen SAXS peaks did not significantly differ at and around the ligation sites compared with those of the normal nerve, whereas the intensities were weaker (Fig. 3).

DISCUSSION

We tried to demonstrate the changes in microstructure after nerve tissue ligation injury by using the SAXS technique. We measured the changes in the microstructure of ligatured rat sciatic nerves in the ex vivo state at the 4C1 SAXS 1 beam line at the PLS in Korea. At the ligatured nerve site and at points proximal and distal to the ligatured nerve site, we also observed microstructure changes, such as changes in the myelin sheath, interfibrillar distance and collagen fiber, that
were caused by the nerve injury.

For comparison to the microstructure changes of ligatured nerve, we measured the horizontal and vertical SAXS pattern of a normal nerve. Our results for the normal sciatic nerve showed 18 nm periodic patterns in the horizontal direction, which represents the myelin sheath; these patterns are caused by the double membrane of the myelin sheath [8]. In addition, 66 nm periodic patterns were measured in the horizontal direction, which represents the interfibrillar space between the longitudinal collagen fibrils. These peaks were wider and were weak [9]. In the vertical direction, 67 nm periodic patterns were measured, and the odd-numbered peaks in the patterns had large widths and high intensities. These represent collagen fibrils, which have a regular structure and are the main component of the nerve membrane [10]. Collagen fibers are formed from 300 nm collagen micro-fibril bundles whose ends have carbon (C-terminal) and nitrogen (N-terminal) [11]. Each end of these has a gap and duplicated space and forms a regular and repeated structure. The broad odd-numbered peaks for the patterns in vertical direction had large intensities, indicating that the gap space is not filled with minerals; instead, the gap is empty. In contrast, broad even-numbered periodic peaks indicate that the gap is filled with inorganic mineral [12].

The myelin sheaths have previously been measured by using the SAXS technique on isolated nerves, such as the frog sciatic nerve or the olfactory nerve [12,13]. Those results showed a basic microstructure that was similar to our results, including the double membrane of the myelin sheath and the periodic patterns of collagen. However, the previous studies did not measure the fine microstructure or the interfibrillar space of collagen.

In the ligatured sciatic nerve, the SAXS patterns were quite different from the normal nerve pattern. The ligatured site did...
Fig. 2. SAXS images of the normal (A) and ligated rat sciatic nerve at the (B) ligation point, (C) 2 mm above the ligation site and (D) 6 mm below the ligation site. At the ligation point, the periodic SAXS patterns of myelin sheath were almost imperceptible, indicative of the loss of the myelin sheath structure. At the points 2 mm above and 6 mm below the ligation site, the myelin sheath peaks became weak and appeared at different q-values, suggestive of disorganization of the bilayer structure of the myelin sheath.

Fig. 3. One dimension SAXS profiles at, above and below the ligation point, displayed with that of normal nerve. The denaturation of myelin sheath structure was observed below and above the ligation point, while total destruction of the myelin sheath structure was seen at the ligatured site and the peak of interfibrillar distance of collagen was weaker at below and above the ligation point but it almost disappeared at the ligation point (A). Vertical periodic peak pattern of collagen at ligatured site, 2 mm above and 6 mm below the ligation site were displayed with that of normal nerve and it observed that there were not different compared with normal pattern but the peaks of ligature site were weak (B).
not show clear periodic patterns in the horizontal direction. This finding indicates complete destruction of the myelin sheath and the absence of interfibrillar space in collagen. However, when measured in the vertical direction, periodic peak patterns were observed for the collagen fibril in the ligatured nerve, and these patterns were lower and weaker than the normal pattern. This result shows that the collagen fibers, which provide structure to the membrane, were not completely damaged. We wanted to compare our results with the results of other histologic observations and analyses of nerve tissue injury, but we could not find any other published studies.

Furthermore, the periodic pattern at the ligatured site differed from the patterns measured 2 mm above and 6 mm below the ligatured site. In the SAXS pattern of the point 2 mm above the ligatured site, the measured patterns of periodic peaks were weak in the horizontal and vertical directions. This finding indicates that the myelin sheath, interfibrillar space and collagen fibers were damaged. Our results were consistent with other histologic observations and analyses of nerve tissue injury. Kim et al. [14] reported that at a position just proximal to the ligatured site of rat sciatic nerve, the fibers were not readily identifiable due to inflammation. Munger et al. [15] reported that 1 cm or more proximal to the constriction, both the myelinated and unmyelinated axons were all normal for a sciatic nerve that was subjected to a constriction injury. Nearer to the constriction, extensive degeneration of myelinated axons became increasingly common, as did signs of endoneurial edema. Beirowski et al. [16] and Raff et al. [17] reported that some retrograde degeneration also occurred but only extended for a few intermodal segments. Thus, it is clear that our results showed that retrograde degeneration occurs above the ligatured site. This finding provides strong evidence that some retrograde degeneration also occurs but only extends for a few intermodal segments.

In the SAXS pattern that was measured 6 mm below the ligatured site, the periodic peaks were weak when measured in the horizontal and vertical directions. This observation indicates that the myelin sheath, interfibrillar space and collagen fibers were damaged. These results were consistent with the results of other histologic observations and analyses of nerve tissue injury. Kim et al. [14] reported that the region distal to a ligatured site showed infiltration of cells with a blue nucleus as a result of Wallerian degeneration. Beirowski et al. [16] and Raff et al. [17] reported that the portion of the nerve fiber distal to the site of injury degenerates because of interrupted axonal transport. Munger et al. [15] reported that the nerve was uniformly edematous and full of myelinic degeneration at the site distal to the constriction. Furthermore, Basbaum et al. [18] reported a near complete loss of large myelinated fibers distal to the ligatures. Many of the surviving axons were shrunken and distorted, although the axons were still in contact with Schwann cells. Thus, it is clear that our results are representative of how nerve damage occurs below the ligatured site.

The schematics for SAXS are illustrated in Fig. 1. SAXS uses angles less than 2° (2θ) and thus requires well-collimated slit systems, generally a 3-slit collimation system, and a long sample-to-detector distance ranging from a few to several meters. Furthermore, 2D CCD detector makes it possible to perform in situ experiments due to the short measurement time and improvement in signal-to-ratio of the data gained by circularly averaging the 2D SAXS image. The SAXS intensity is the square of scattering amplitude, |A(q)|², and it can be presented as

\[ I(q) = |A(q)|^2 = \int_0^\infty \rho(x) e^{-iqx} dx \]

where q is scattering vector defined as \( q = k_i - k_s \) and \( \rho(t) \) is the electron density. The vectors \( k_i \) and \( k_s \) are the incoming and scattered wave vectors, respectively, and are defined as \( k_i = 2\pi/\lambda \), where \( \lambda \) is the X-ray wavelength. In the SAXS profile, the scattering vector q has the following relationship with the scattering angle (θ): \( q = (4\pi/\lambda) \sin \theta \). From Bragg’s law, the periodic layer spacing (d-spacing) is calculated as \( d = 2\pi/\lambda \).

In this study, we address periodic nanostructures; therefore, a regularly arranged layer spacing (d spacing) can be determined using only the above equation.

For our study, we used the sciatic nerve because the soma of axons in peripheral nerves is less complex and easy to dissect [1]. The myelin sheath is composed of water (40%), lipid (42–51%) and protein (9–18%), and it has a fluid-crystalline or liquid-crystalline nature [19]. Thus, the structure in most nerves might be deformed by mechanical and chemical processing for experiments because of the liquid-crystalline nature at room temperature [20]. Therefore, we tightly sealed the fragment of the nerve between polyamide films to prevent evaporation of the water component and deformation of the nerve by using polyamide films.

The SAXS technique provides structural information about organs without sample preparation [4,5]. However, in many
studies, chemical or mechanical nerve preparations were used [9,12,13]. We removed the nerve fragments at the beam line just before the SAXS measurement and did not utilize chemical or mechanical nerve preparations.

To develop a neuropathic pain model, three models have commonly been utilized: (1) the chronic constriction injury by the loose ligation of the sciatic nerve model of Bennett and Xie [21], (2) the tight ligation of the partial sciatic nerve (PSL) model of Seltzer et al. [22] and (3) the tight ligation of spinal nerves model of Kim and Chung [23]. These different methods are useful neuropathic pain models [24]. We used the PSL model to induce nerve damage.

We could not obtain results from sites that were more proximal site than 2 mm or more distal than 6 mm from the ligatured site because of limitations in the nerve fragment.

From a clinical point of view, traumatic disorders, such as nerve, spinal cord, or head injury, are not the only cause of Wallerian degeneration. Instead, it is now broadly accepted that Wallerian degeneration is mechanistically related to nerve fiber loss in many neurodegenerative disorders, such as amyotrophic lateral sclerosis and multiple sclerosis [25,26]. Therefore, it is clinically important to obtain detailed microscopic imaging at various sites near nerve lesions of varied severity during the early post-injury phase. We obtained very detailed images of the morphologic changes in ligatured nerve fibers using the SAXS technique at the ligatured site, proximal and distal to the ligatured site. Our SAXS results indicate that the present SAXS technique may provide a new imaging modality for clinical applications in the biological and medical sciences.

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