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AN ULTRASTRUCTURAL MODEL TO TEST MICROBURST STIMULATION OF NERVES

An Undergraduate Research Scholars Thesis

by

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TABLE OF CONTENTS

PAGE

TABLE OF CONTENTS ……………………………………………………………………1

ABSTRACT …………………………………………………………………………….2

ACKNOWLEDGEMENTS ………………………………………………………………4

CHAPTER

I INTRODUCTION ……………………………………………………………5

II MATERIALS AND METHOD …………………………………………………13

III RESULTS AND CONCLUSIONS ………………………………………….…..13

Formalin recovery technique feasibility …………………………………………13
Increased mean myelin area in recovered tissues ……………………………….17
Trends similar in recovered and control tissues …………………………………25
Mean g-ratio unchanged in recovered tissues …………………………………...26
Conclusion ……………………………………………………………………29

REFERENCES ……………………………………………………………………….31

ABSTRACT

An Ultrastructural Model to Test Microburst Stimulation of Nerves. (May 2013)

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Some patients that suffer from epilepsy may become refractory to pharmaceutical treatment. An option with these patients is vagus nerve stimulation (VNS) therapy with neuro-cybernetic medical devices. The purpose of this research is two-fold: 1.) to determine if a recovery technique can be used with formalin-fixed samples of nerve tissue for transmission electron microscopy (TEM) and 2.) to determine if there is an ultrastructural difference in tissue exposed to the neuro-cybernetic device. If successful, the study will reduce animal studies and expense by establishing a mechanism to perform retrospective TEM studies on formalin-fixed tissue. Additionally, TEM allows examination of specimens in much greater detail than light microscopy. Therefore, using TEM to compare ultrastructural differences in tissue that was exposed to the medical device and healthy tissue will help determine with precision if any damage is caused by the medical device. To complete these objectives, formalin-fixed vagus nerve tissue from goats that were exposed to the medical device is collected, recovered, evaluated by TEM, and compared to healthy traditionally fixed vagus nerve tissue. Results show that the recovery technique makes it possible to achieve quantitative data from formalin-fixed tissue samples. This method establishes a mechanism to execute retrospective TEM studies on formalin-fixed tissue, thereby reducing future animal studies. Results also show that there are

some differences in goat nerve that has been exposed to the medical device. These subtle ultrastructural changes (potentially reversible) do not appear to have clinical impact.

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Additionally, I would like to acknowledge all of the people at the Cardiovascular Pathology Laboratory, Texas A&M University. Throughout this process, the students and faculty working in this lab always provided me with a friendly face and kind words.

CHAPTER I

INTRODUCTION

Epilepsy is a neurological disorder that affects about 50 million people of all ages and all nations.[1] Epilepsy is characterized by a predisposition of the brain to generate sudden, recurrent seizures.[2] Epilepsy is not a singular condition; patients suffering from a variety of disorders involving irregular brain functioning seizures are considered to have epilepsy. An epileptic seizure can be defined as a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain.[2] Seizures vary widely in nature of onset, duration, severity, clinical manifestation, and frequency. Their cause is often idiopathic. Approximately 70% of epileptic patients are effectively treated with anti-epileptic medications. For the 30% of epileptic patients that become refractory to pharmaceutical treatment, research is needed to discover an effective alternative method.[1]

Vagal Nerve Stimulation (VNS) therapy is one proposed adjunctive therapy for reducing seizure frequency in adults and adolescents over 12 years of age that experience partial onset of seizures and are refractory to anti-epileptic drugs. Today, over 50,000 patients in over 70 different countries use vagal nerve stimulation therapy.[3] Various studies indicate that this sort of stimulation may be beneficial in treating long term or chronic depression, chronic anxiety, bulimia, Alzheimer's disease, and chronic migraines in addition to epileptic therapy. VNS therapy involves a neuro-cybernetic prosthesis (NCP) that sits in the chest of the patient. Electrodes extending from the device make contact with the vagus nerve and provide varying levels of microburst, electrical stimulation.

Previous studies indicate that electrical stimulation may cause damage to the myelination of verve tissue. Myelin is a vital insulating component for signal conduction in nerve tissue. Research completed in 1999 using cat nerve tissue found that continuous high frequency electric stimulation causes nerve degeneration due to excessive neural activity.[4] Additional studies using the peroneal nerve in cats concluded similar results: prolonged high frequency stimulation caused irreversible nerve damage, while prolonged low frequency electrical stimulation showed no morphological changes to the nerve. Studies have found that application of an interrupted high frequency stimulus produces less damage than application of a constant high frequency stimulus.[5] Current VNS innovation involves microburst stimulation, which is characterized by short bursts or pulses of stimulation separated by brief periods without stimulation. By varying the stimulation parameters, including frequency and duration of the stimulus, researchers hope to find an intermediate level of stimulation that can be used for safe and effective treatment of a variety of medical conditions.

To evaluate improvement of stimulation parameters or device design, histology of nerve tissue must be closely examined. Vagus nerve stimulation causes no measureable physiologic changes. Light microscopy is a common histological evaluation tool. Transmission electron microscopy views at a much greater magnification, allowing more detail of ultrastructural components. By measuring the area of the myelin sheath in the nerve, quantitative data can detect minute nerve changes that occur in response to changes in electrical stimulation parameters or device mechanics, allowing a better evaluation of the NCP.

TEM requires specific fixation, handling, and sectioning to preserve the detail of the specimen. Formalin is a common fixative for histology and works well for light microscopy, however, formalin fixation is not acceptable for TEM. Standard fixation for TEM uses Karnovsky's solution which consists of glutaraldehyde and freshly prepared paraformaldehyde in a buffer suitable for pH of 7.0-7.4. Tissue from this study was used in a previous, unrelated study and had already been fixed with formalin. Therefore, a unique procedure was used to recover this formalin-fixed tissue so that it could be used for TEM evaluation. Results from this study will determine the feasibility of this formalin recovery technique as compared to the standard Karnovsky's method. If feasible, this technique will allow retrograde TEM studies to be performed on formalin-fixed tissue, reducing future animal studies and expense.

The purpose of this research is two-fold. The first part will focus on determining the feasibility of the formalin recovery technique for evaluation of vagal nerve tissue with transmission electron microscopy. The second part will determine if there is an ultrastructural difference in tissue exposed to the neuro-cybernetic device and healthy, goat vagal nerve tissue. If successful, this study will establish a mechanism to do retrospective TEM studies on formalin-fixed tissue, thereby reducing future animal studies. Additionally, success in comparison of this tissue with control tissue will provide information about the effects of VNS therapy in NCP devices to the ultrastructural components of nerve tissue. This information can be applied to help determine the safety and efficacy of this device in treating human patients with refractive epilepsy.

Vagus nerve tissue is slightly different in size, composition, and myelinated area among different species. To successfully apply information learned in this study to human studies, it must be

established that goat tissue is a good model for human tissue. Goat vagus nerve tissue is similar in length, diameter, and composition to human vagus nerve tissue and therefore, is a sufficient model for quantitative data collection comparisons. Healthy vagus nerve tissue studies have been performed in the cat, mouse, ferret, rat, lamb, human, and goat.[6-12] Results from this study will be compared to results from a study using healthy goat vagal tissue (standard fixation). Since vagal goat tissue is similar in size and composition to human vagal nerve tissue, this study can provide a sound basis for accurate predictions of how human vagal nerve tissue would respond to electrical stimulation, tissue processing with the formalin recovery technique, and evaluation using transmission electron microscopy.

CHAPTER II

MATERIALS AND METHODS

Tissue for this study was harvested from four adult goats in a tissue sharing initiative from an unrelated study. This study was approved by the Texas A&M University, Animal Care and Use Committee. Collections were performed at the Translational Pathology Research Laboratory at Texas A&M University.

Each goat was implanted in the chest with a neuro-cybernetic prosthesis (NCP) system and received microburst stimulation to the left vagus nerve. Two of the goats were implanted with a sham device that did not produce electrical stimulation on the right vagus nerve. The device remained in one goat for 21 days and remained in the other three goats for 36 days. Each of the four goats received slightly different microburst stimulation patterns at the left vagus nerve. The right vagus nerve received no stimulation. After the testing period, the goats were euthanized and tissue removed to examine the effects of the device.

Tissue was harvested within ten minutes of the time of anesthetization. Carotid arteries were first flushed with physiologic saline and then perfusion fixed with 10% neutral buffered formalin. A block of tissue containing the device, skeletal muscle, and the lateral groove composed of the carotid artery, carotid vein, and vagus nerve was removed from each animal and placed in 10% neutral buffered formalin for additional fixation. Fat and vascular tissue were carefully sectioned away, leaving only vagus nerve.

Six nerves were harvested in total. In two of the goats, both the left (exposed to NCP device) and right (exposed to sham) vagus nerves were harvested; in the other two animals, only the left vagus nerve was harvested. Four, approximately 1-2cm samples were taken from each nerve, making a total of 24 tissue samples. Samples were taken at the following locations in relationship to the device: 4cm cranial, 2cm cranial, 2cm caudal, and 4cm caudal.

A special technique was used to recover formalin-fixed tissue into samples that can be viewed using transmission electron microscopy. Sections were fixed at room temperature in modified Karnovsky's (2% (vol/vol) glutaraldehyde: 2% (wt/vol) paraformaldehyde in 0.1 M HEPES buffer pH 7.4) fixative overnight, rinsed in 0.1 M HEPES buffer, pH 7.4, post-fixed overnight in 1% osmium tetroxide, 0.1 M HEPES buffer, pH 7.4.[13] Dehydration was done in a closely graded methanol series (5% steps (vol/vol) from 5% to 100%) with 1% (wt/vol) paraphenylenediamine to improve stain contrast. Samples were transferred to propylene oxide, then infiltrated and embedded using a continuous addition of epoxy resin in small increments over a period of seven days. Samples were kept on a rotator between additions of resin. A unique resin recipe was used for the epoxy resin. To make 20 grams of resin, use 3.54 g Queotol, 2.92 g LX-112, 13.54 g DDSA, and 0.5 mL of Benzyldimethylamine (BDMA). Following embedding, blocks were trimmed for sectioning.

Sectioning was done on a Sorrall MT600 ultramicrotome using a diamond knife. Ultra thin sections were cut from the outer perimeter of the nerve tissue. Sections were cut approximately 120 nm thick and placed on 150 mesh grids. Ultra thin sections were post-stained with 2% aqueous uranylacetate followed by Reynold's lead citrate (Reynolds 1963).[14]

Electron micrographs (approximately 10-12 per tissue sample) were captured at 5,000x magnification. Images were processed by hand using Image J software to measure the area of each axon and the area of the myelin sheath (calculated by subtraction of the area of the axon from the area of the entire fiber). The number of the myelinated axons present per field was counted. Due to poor quality of some of the images, axons in which the edges of axons could not be easily distinguished were not included in the data. Only axons that lie completely within the field were counted and measured.

Images and morphometric data from a study using healthy goat vagus nerve tissue will be used as a control to compare with data collected in this study. Tissues from the control study were immediately fixed with the standard TEM fixative, Karnovsky's solution (2% (vol/vol) glutaraldehyde: 2% (wt/vol) paraformaldehyde in 0.1 M HEPES buffer pH 7.4) fixative overnight. Samples were rinsed in 0.1 M HEPES buffer, pH 7.4, post-fixed overnight in 1% osmium tetroxide, 0.1 M HEPES buffer, pH 7.4, and dehydrated with a closely graded methanol series (5% steps (vol/vol) from 5% to 100%) with 1% (wt/vol) paraphenylenediamine to improve stain contrast, exactly as samples from this study. Sectioning methods and manual data collection for control samples were consistent with those used in this study.

Tissue from the present study will be divided into two groups for comparison: 1.) those that received electrical stimulation (left tissue samples) and 2.) those that were exposed to the sham device and received no stimulation (right tissue samples). These two groups will be compared to control samples. Comparison will identify differences that may be due to fixation technique,

device exposure, or electrical stimulation from VNS therapy. Table 1 shows a summary of the comparison groups.

Table 1: Summary of Comparison Groups			
	Control Group	**NCP Device**	**Sham Device**
Tissue Collection	Previous Study	Left Samples	Right Samples
Fixative	Karnovsky's	Formalin, then Recovered	Formalin, then Recovered
Electrical Exposure	No	Yes	No
Mechanical Exposure	No	Yes	Yes

CHAPTER III

RESULTS AND CONCLUSIONS

Formalin recovery technique feasibility

It can be concluded that the formalin recovery technique used in this study is a feasible way to process tissue so that it can be evaluated using TEM. Formalin is not a fixative for TEM, but this technique allows vagal nerve tissue to be viewed at the ultrastructural level. TEM allows both qualitative and quantitative data of myelinated nerves to be gathered. Successful use of this technique provides a mechanism to do retrospective TEM studies on formalin-fixed tissue, thereby reducing future animal studies and expense.

Morphology using TEM

Nerves were harvested for this study based on fixation (formalin and modified Karnovsky's) and electrical stimulation. A non-statistical side by side comparison is used to compare morphology between healthy goat vagal nerve tissue (standard fixation) and goat vagal nerve tissue that was exposed to the NCP device (formalin-fixed and recovered). Figure 1 shows myelinated neurons prepared by standard fixation for TEM. Figure 2 is an image of myelinated nerve prepared by the formalin-fixed recovery technique that starts with formalin fixation and is later treated with the aforementioned Karnovsky's solution. Both of these two techniques demonstrate identifiable myelinated nerves. However, the Karnovsky's fixed tissue provides better resolution of subtle degranulation (vacuolation change) (dark arrow). In contrast, the formalin recovery technique shows better definition of the substrate collagen fibrils (white arrow). Both techniques had

comparable results as to qualitative identification of myelin. The second portion of the evaluation is to compare the myelinated nerve quantitatively.

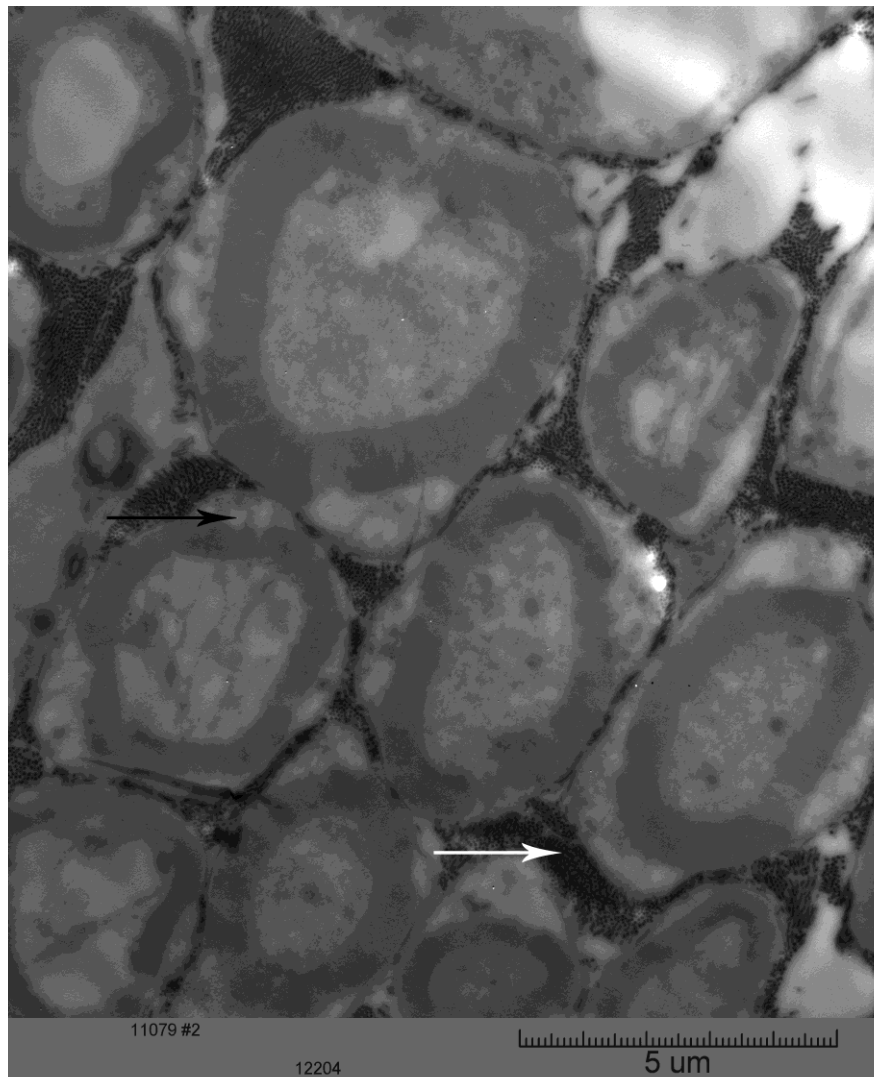

Figure 1- ***TEM Cross Section of Recovered (formalin-fixed) Myelinated Nerve*****:** Representative micrograph of the vagus nerve showing myelinated fibers, vacuolation (dark arrow), and collagen substrate (white arrow) at 5,000x magnification. Partial neurons (as seen on the edges of the image) were not included in data collection.

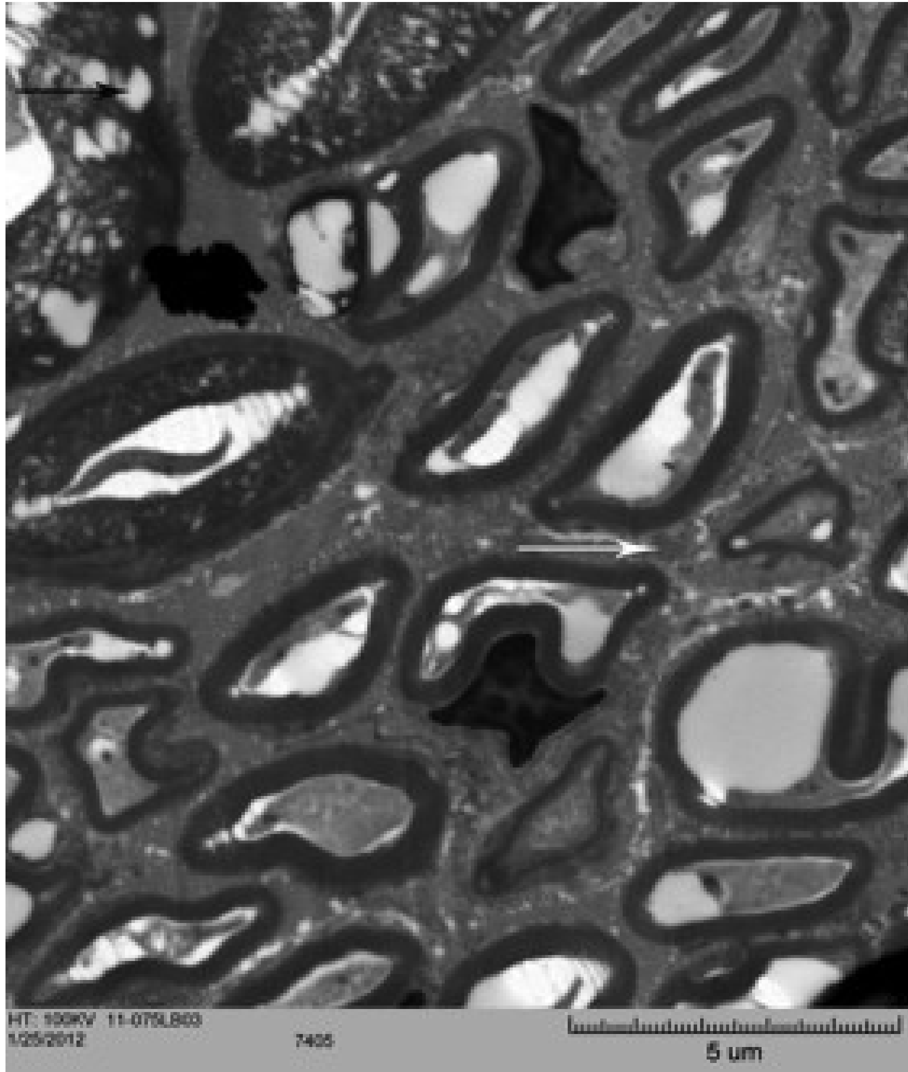

Figure 2- ***TEM Cross Section of Standard Karnovsky's Fixed Myelinated Nerve:*** Representative micrograph of the vagus nerve showing myelinated fibers, vacuolation (dark arrow), and collagen substrate (white arrow) at 5,000x magnification. Partial neurons (as seen on the edges of the image) were not included in data collection.

Morphometrics using TEM

Morphometrically, there were notable differences in myelin area among electrically stimulated (left vagus), sham (right vagus), and control vagal nerve tissue. Simple comparison between recovered tissue samples (left and right) and control tissue samples showed notable differences. Measurements of the myelin area for recovered tissue samples showed a skewed normal

distribution around a mean of 5.92 um^2 with a standard deviation of 4.16 (Figure 3). Analysis of goat vagal nerve tissue in the control study showed a mean myelin area of 3.79 um^2 with a standard deviation of 0.07 um^2(Figure 4).[6]

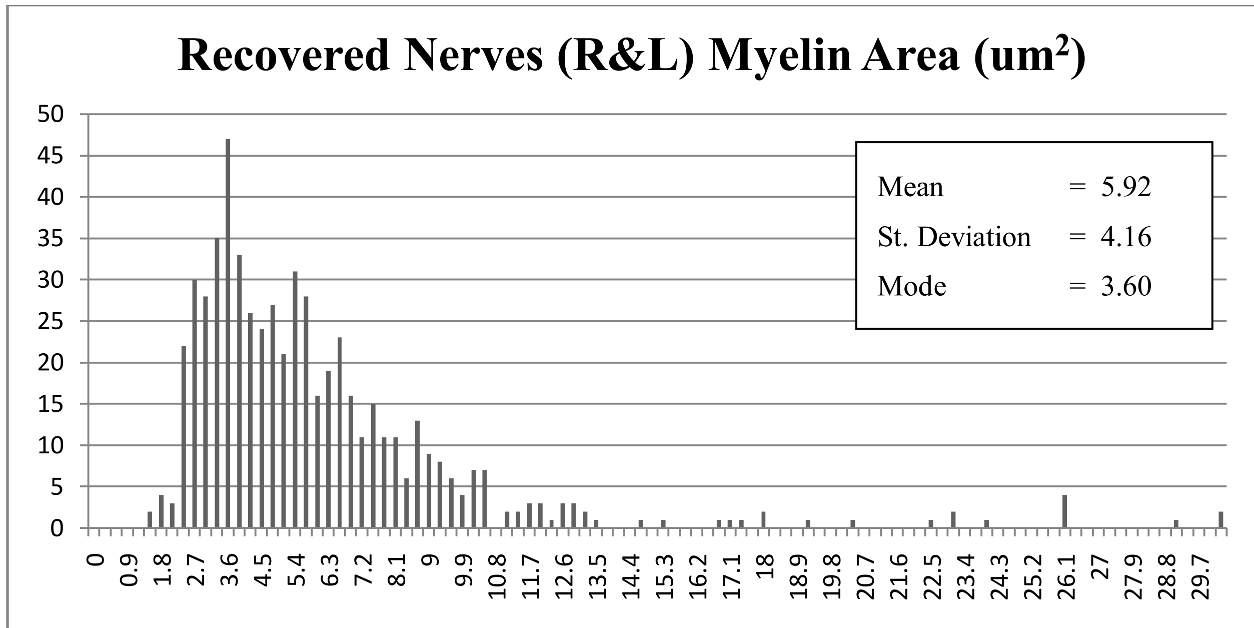

Figure 3- ***Recovered Nerves (R&L) Myelin Area(um^2):*** Histogram shows myelin areas (um^2) of all axons in formalin-fixed tissue samples taken from both right and left sides of animals that were implanted with the NCP medical device. Axons that were incomplete or partially contained outside the image capture were not included. n=583 axon measurements/6 animals

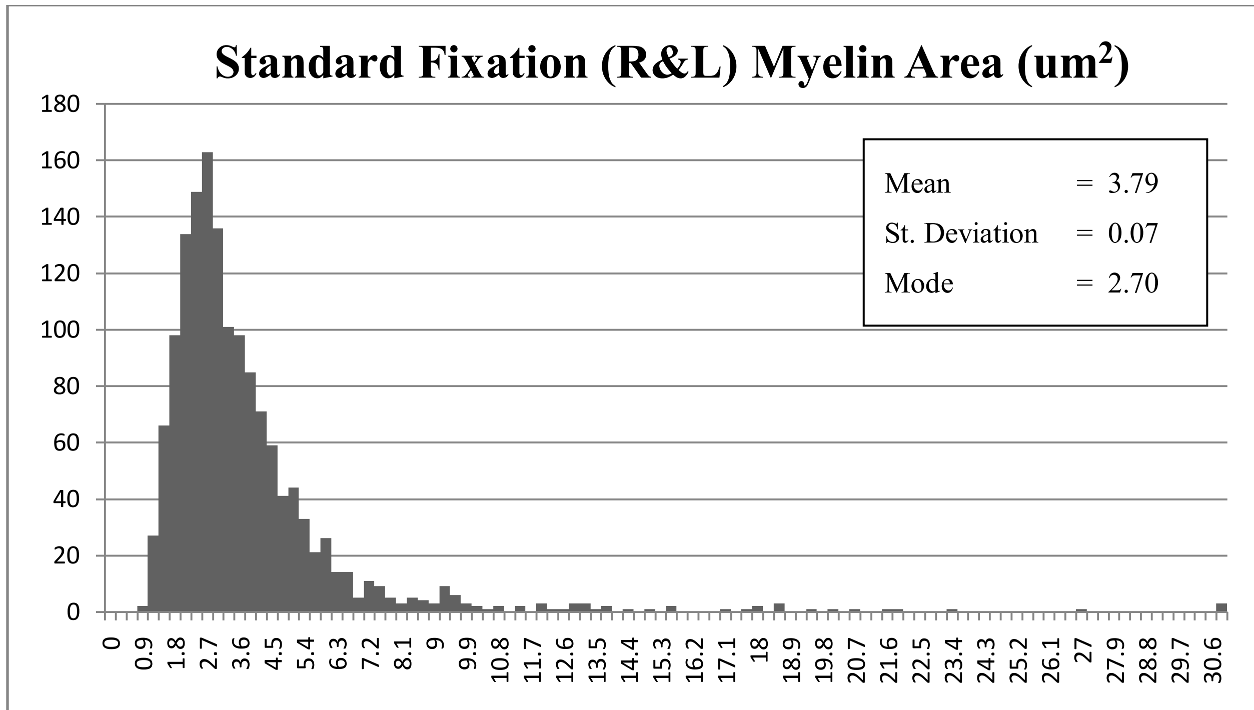

Figure 4- ***Standard Fixation (R&L) Myelin Area (um²):*** Histogram shows myelin areas (um²) of all axons in traditionally fixed tissue samples taken from both right and left sides of animals that were never exposed to a medical device. Axons that were incomplete or partially contained outside the image capture were not included. This data will be used as a control. n=1490 axon measurements/9 animals

Increased mean myelin area in recovered tissues

Recovered tissue (both left and right sides) had mean myelin areas that were much higher than the baseline study. This might indicate reversible damage in the form of edema and swelling was caused by electrical stimulation, mechanical presence of the device, or surgical handling. To delineate this cause, the recovered right and left tissue samples are compared separately.

Mean myelin area comparison

Recovered tissue samples from left and right sides were evaluated separately to determine the source of altered myelin area. Both left and right groups showed skewed normal distribution. Because of inherent differences in the anatomy of left and right vagal nerves in healthy animals,

to make appropriate comparisons, right nerves should be compared only to healthy right nerves and left nerves should be compared only to healthy left nerves.[6] Recovered tissue samples that were exposed to electrical stimulation (left samples) have a mean myelin area of 5.69um^2 and a standard deviation of 4.19 um^2 (Figure 5), compared to the control left nerves that have a mean of 3.51um^2 and a standard deviation of 2.34 um^2 (Figure 6). Recovered tissue samples that were exposed to the device but received no stimulation (right samples) have a mean myelin area of 6.39um^2 and a standard deviation of 4.04 um^2 (Figure 7), compared to the control right nerves that have a mean of 4.35um^2 and a standard deviation of 4.19 um^2 (Figure 8).

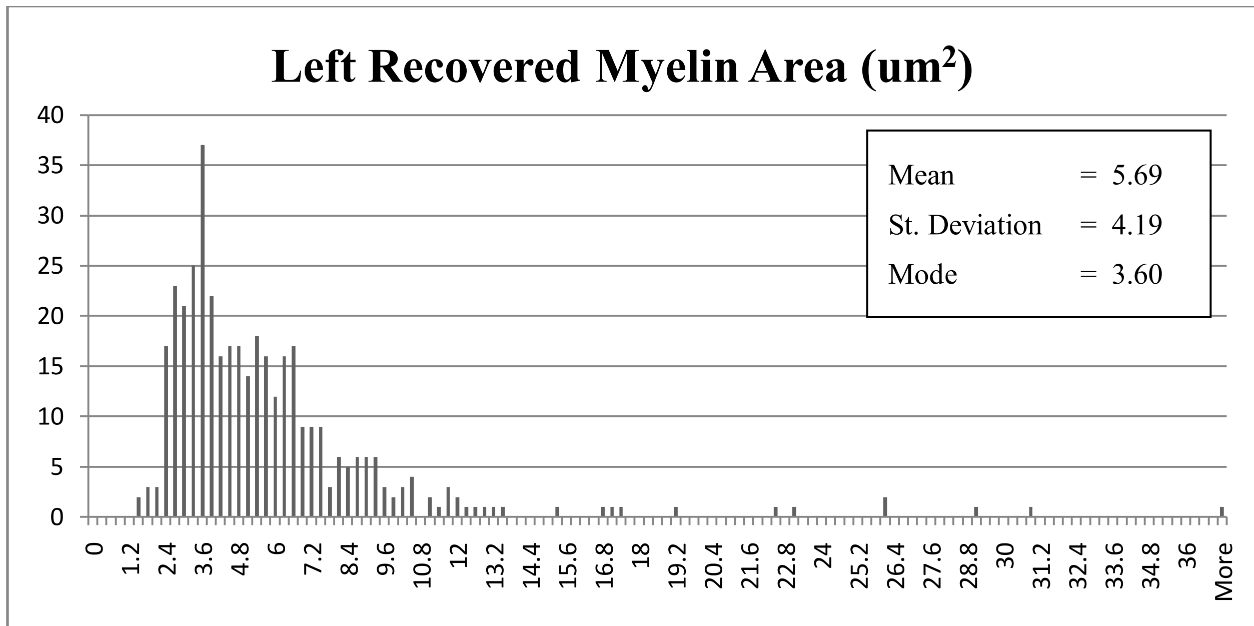

Figure 5- ***Left Recovered Myelin Area (um^2):*** Histogram shows myelin areas (um^2) of all axons in formalin-fixed tissue samples taken from left sides of animals that were implanted with the NCP medical device and received varying levels of electrical stimulation from that device. Axons that were incomplete or partially contained outside the image capture were not included. n=392 axon measurements/4 animals

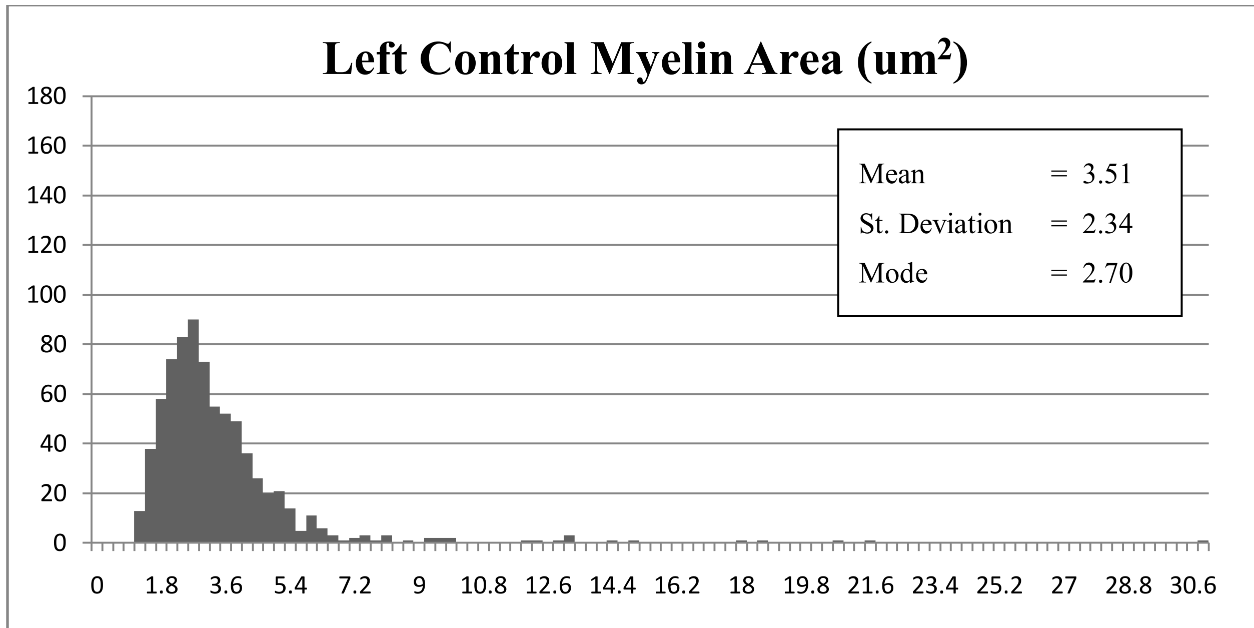

Figure 6-*Left Control Myelin Area (um^2):* Histogram shows myelin areas (um^2) of all axons in traditionally fixed tissue samples taken from left sides of animals that were never exposed to a medical device. Axons that were incomplete or partially contained outside the image capture were not included. This data will be used as a control. n= 757 axon measurements/4 animals

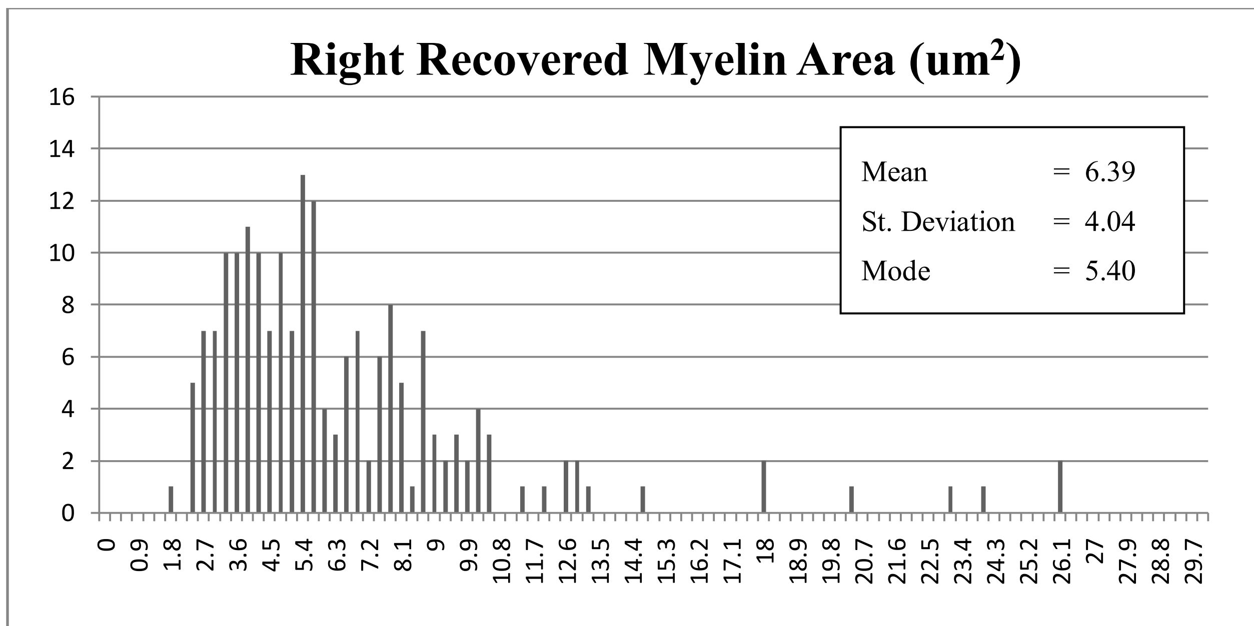

Figure 7- *Right Recovered Myelin Area (um^2)*: Histogram shows myelin areas (um^2) of all axons in formalin-fixed tissue samples taken from right sides of animals that were implanted with the NCP medical device but did not receive electrical stimulation from the device (sham). Axons that were incomplete or partially contained outside the image capture were not included. n=191 axon measurements/2 animals

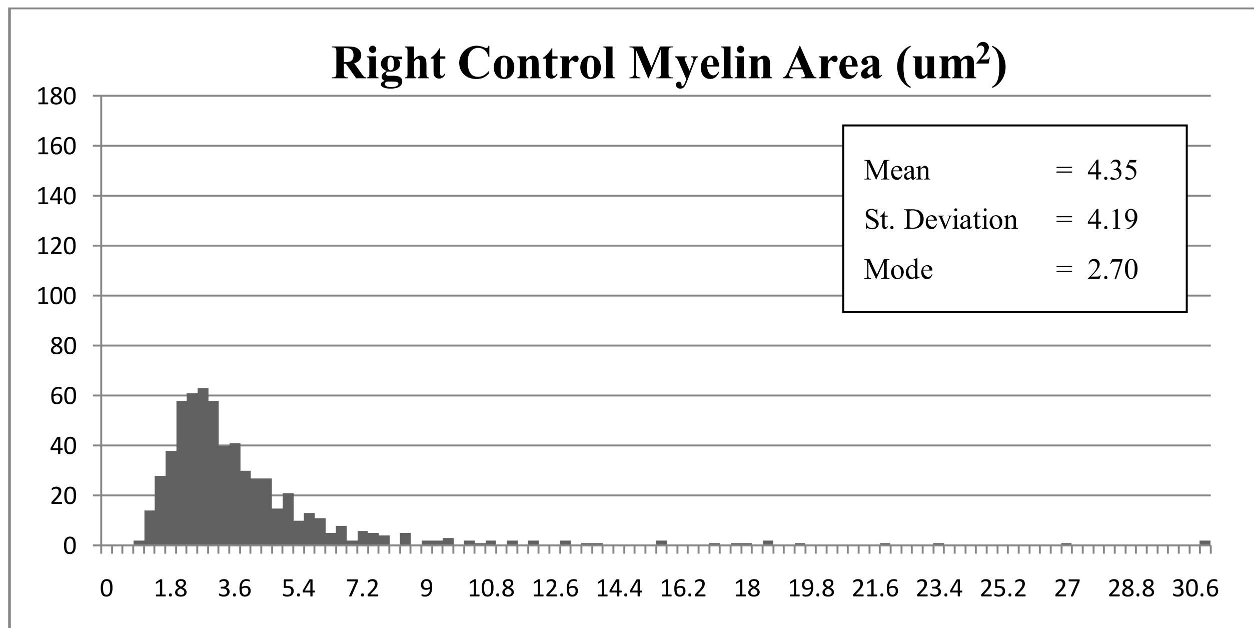

Figure 8- ***Right Control Myelin Area (um^2):*** Histogram shows myelin areas (um^2) of all axons in traditionally fixed tissue samples taken from right sides of animals that were never exposed to a medical device. Axons that were incomplete or partially contained outside the image capture were not included. This data will be used as a control. n=733 axon measurements/5 animals

Standard deviation comparison

Standard deviation of mean myelin area for left recovered tissues is much higher (not statistically significant) than standard deviation of mean myelin area of left control tissues. Standard deviation of mean myelin area for right recovered samples is about the same as standard deviation of mean myelin area of right control samples.

Left recovered tissue samples received varying levels (current and duration) of electrical stimulation which could explain the wide spread of data (increased standard deviation) when compared to control tissues. Myelin areas of left nerves are generally very centered around the mean (as seen in the control left tissue samples) but varying levels of electrical stimulation may

have caused the myelin areas to swell and shrink in response to short period of exposure or a long period of exposure respectively.

Standard deviation of right recovered tissue samples was consistent with that of right control tissue. Presence of mechanical device may have caused an increase in mean myelin area, but spread (standard deviation) of myelin area was not notably altered. Contrary to the left tissue samples, in which some tissue samples shrank and some swelled, in the right tissue samples, the entire histogram seems to have shifted to the right, indicating that all neurons experienced a similar increase in myelin area. This is consistent with expectations since all of the right tissue samples were treated the same (were exposed to the device but received no electrical stimulation) while left tissue samples were treated slightly differently in regards to the duration of electrical stimulation.

Mode comparison

The mode of myelin area may be a better indicator of changes than the mean myelin area. The mode of myelin area for all recovered tissue samples (right and left) is 3.60um^2; this is much closer to 3.79 um^2, the mean myelin area of tissue samples from the control study (Figures 3 and 4). This finding indicates that although some of the myelinated areas were affected by the device or electrical stimulation from that device, there were a notable number of axons that were almost completely unaffected by the stimulation; a large number of axons had a mean myelin area that remained consistent with mean myelin areas of healthy vagal nerve tissue.

The mode of myelin areas for left recovered tissue samples is 3.60um^2 and is very close to 3.51um^2, the mean myelin area of left control tissue samples (Figures 5 and 6). There were 392 data pieces taken for left tissue samples and 37 of them fell in the 3.60 um^2 bin, indicating that approximately 9% of the neurons had myelin areas that were consistent with the mean myelin area of control data. While many of the left neurons did swell after being exposed to electrical stimulation, 9% did not increase or decrease in size. The mean myelin area may be falsely inflated because of the axons that had considerably increased mean myelin areas.

The mode of myelin areas for recovered right tissue samples is 5.40um^2 with 13 of the 191 data pieces falling into this bin (Figures 7 and 8). The mode for right recovered samples is not as important as the mode for left recovered samples because right data shows several measures with high frequency. Myelin areas of 3.30 um^2, 3.60 um^2, 3.90um^2, 4.20um^2, 4.80um^2, and 5.70um^2 all have frequencies of ten or more (Figure 7). This is normal since recovered right data had a larger spread (standard deviation) than recovered left data. A comparative summary of mean, standard deviation, and modes of the myelin area of recovered tissue samples and from control tissue samples is shown in Table 2.

Table 2: Summary of Myelin Area				
	Mean	**St. Deviation**	**Mode**	**# Data Pieces**
Right Recovered	6.39	4.04	5.40	191
Right Control	4.35	4.20	2.70	733
Left Recovered	5.69	4.19	3.60	392
Left Control	3.51	2.34	2.70	757
R&L Recovered	5.92	4.16	3.60	583
R&L Control	3.79	0.07	2.70	1490

Stimulation comparison

Stimulation parameters for the left recovered samples varied in current and duration. Although all left samples have mean myelin areas that are considerably higher than the control samples, there is some variation among the goats that may be attributed to varying microburst stimulation. Two of the goats, animal number 73 and 79, had mean myelin areas of 5.47um^2 and 5.32um^2 respectively. The other two goats, animal number 78 and 80, had mean myelin areas of 6.15 um^2. Based on this data, it is expected that the goats with the larger increase in mean myelin area (78 and 80) received a higher frequency of current, more current, or longer stimulation. This study found no consistent connection between current, duration, and myelin area and more research will have to be done to investigate these hypotheses. Table 3 summarizes the data gathered and stimulation parameters for each animal.

Table 3: Summary of Left Recovered Tissue Results and Stimulation Parameters						
Animal	**Mean (um^2)**	**St. Dev**	**Mode (um^2)**	**Current (mA)**	**Frequency (Hz)**	**Duration**
73	5.32	3.40	3.30, 3.60	0.25	300	Few min several times a day
78	6.15	5.85	3.50	1.25	300	Pulse width of 250 microsec Interval burst of 0.2sec with 7 pulses
79	5.47	3.70	3.60	1.25	300	Pulse width of 250 microsec Interval burst of 0.2sec with 7 pulses
80	6.15	4.03	4.00	1.25	300	Pulse width of 250 microsec Interval burst of 7200sec on/0.2min off; no pulses

Animal number 73 received less current than the other animals, was exposed to much less frequent stimulation, and was only exposed to the device for 21 days (rather than 36); any of these differences may explain the slightly lower mean myelin area. Animals 78 and 79 received the same microburst parameters, so it is expected that changes in myelin area of tissue samples would be the same for these samples, but they were slightly different. Instead, animals 78 and 80 showed similar results for mean myelin area, even though they received slightly different microburst patterns. More research must be done to make conclusive correlations between microburst treatment and ultrastructural changes in nerve tissue

Location comparison

Recovered tissue samples were taken from four different locations in relationship to the medical device: 4cm cranial, 2cm cranial, 2cm caudal, and 4cm caudal. Table 4 shows a summary of the comparisons of myelin area based on location.

Table 4: Summary of Myelin Area Comparisons Based on Location		
Location	**Mean (um^2)**	**St. Deviation**
4cm Cranial	5.54	3.15
2cm Cranial	5.35	3.26
2cm Caudal	7.51	5.96
4cm Caudal	6.02	4.58

Tissue samples taken cranial to the device had a slightly lower mean myelin area than samples that were caudal to the device. Standard deviations of cranial tissue samples were slightly less (not statistically significant) than caudal tissue samples.

Trends similar in recovered and control tissues

Mean myelin area in left tissue samples was slightly less (not statistically significant) than mean myelin area in right tissue samples for both control and recovered samples. Consistent trends occur in recovered tissues and control tissues, validating the data from recovered tissues. Differences in mean myelin area between recovered and control tissues does not easily allow direct comparison of these tissues. Consistent trends indicate that similar conclusions can be drawn when comparing right and left recovered tissues and right and left control tissues.

A major limitation in direct comparison between data from control vagal nerve tissue and data from recovery tissue was reduced count of axons. The control study using healthy vagal nerve tissue had about 1490 data pieces (including both right and left tissue samples) while the present

study used only 583 data pieces (including both right and left tissue samples). With more images and more axons in each image, it is expected that histograms from the present study would be more similar to those reflecting data collected from healthy nerves.

Mean g-ratio unchanged in recovered tissues

G-ratio is a calculation of axon diameter/fiber diameter that is widely used to determine the optimal axonal myelination for functional and structurally sound nerves. The optimal g-ratio for peripheral nerve tissue is 0.6.[15]

Calculations of the g-ratio of all recovered tissue samples show a normal distribution around a mean of 0.32 with a standard deviation of 0.10 (Figure 9). The control tissue has a g-ratio with a normal distribution around a mean of 0.33 and a standard deviation of 0.90.[6] When divided, the left and right recovered tissue samples showed slightly different means; left nerves had a mean of 0.32 with a standard deviation of 0.11 (Figure 10) while right nerves had a mean of 0.31with a standard deviation of 0.10 (Figure 11). Left and right tissue samples from the baseline study had mean g-ratios of 0.33 with a standard deviation of 0.09.

There is basically no difference in mean g-ratio of: right recovered (formalin-fixed), right control (standard fixation), left recovered (formalin-fixed), left control (standard fixation). Table 5 shows a summary of this data. Varying treatment and fixation did not alter g-ratio. This may indicate that nerves from this study are working as efficiently as nerves in the control study and that no significant damage occurred. However, more research will need to be performed to fully validate this possible outcome. This is noteworthy because although there may be damage that

causes neurons to swell and shrink, as long as the axon diameter and fiber diameter of the neurons are changing proportionally, the nerve is still able to transmit neural signals effectively.

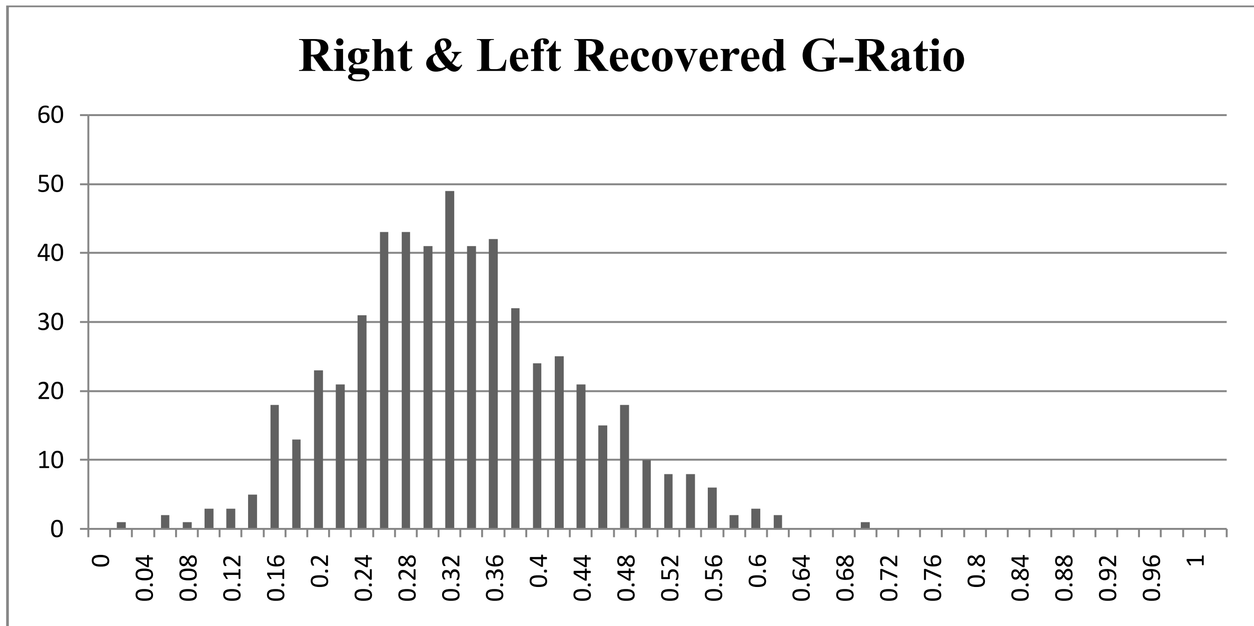

Figure 9- ***Right &Left Recovered G-Ratio:*** Histogram shows g-ratio of all axons in formalin-fixed tissue samples taken from both right and left sides of animals that were implanted with the NCP medical device. Axons that were incomplete or partially contained outside the image capture were not included. n=583 axon measurements/6 animals

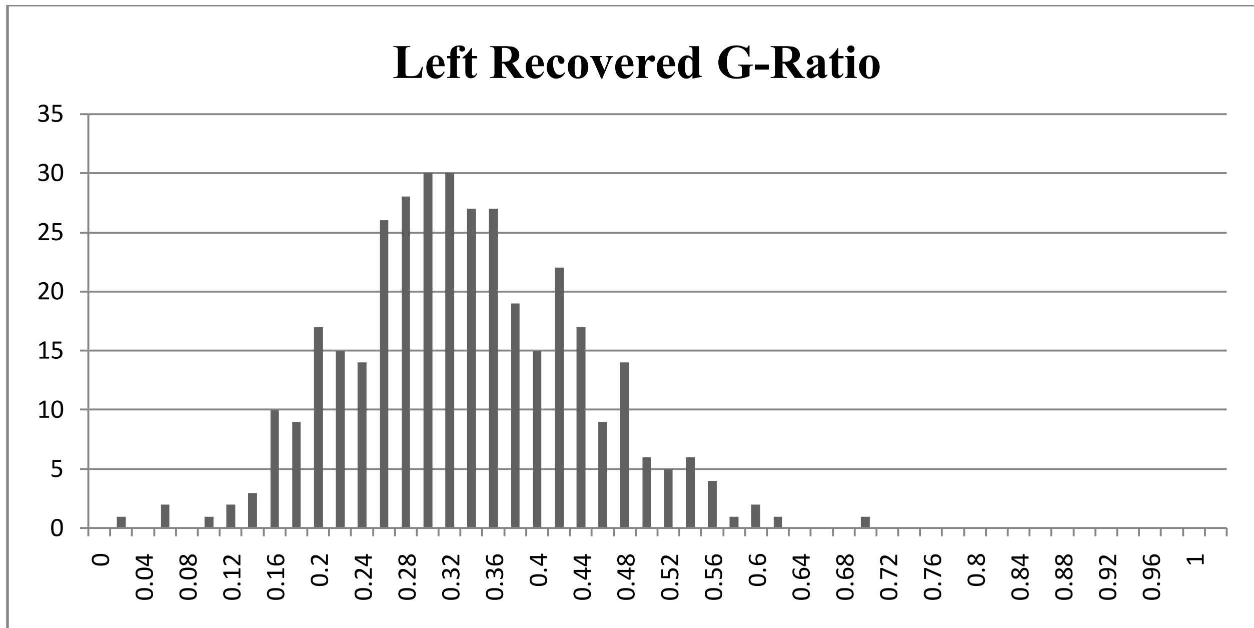

Figure 10- ***Left Recovered G-Ratio:*** Histogram shows g-ratio of all axons in formalin-fixed tissue samples taken from left sides of animals that were implanted with the NCP medical device. Axons that were incomplete or partially contained outside the image capture were not included. n=392 axon measurements/4animals

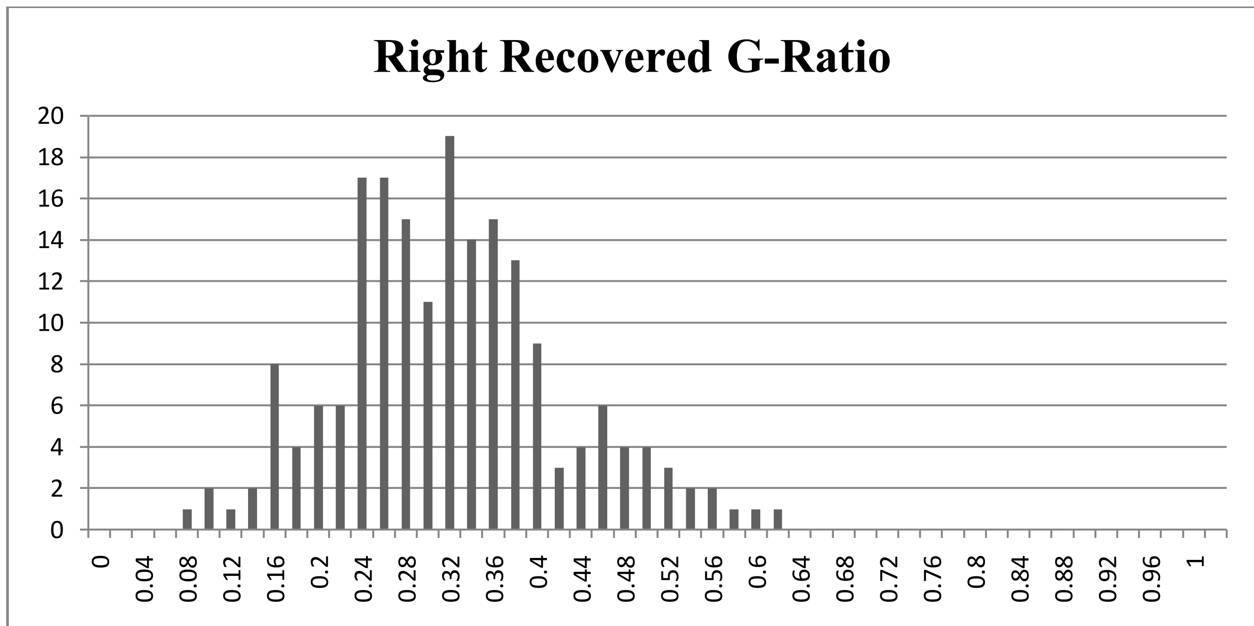

Figure 11- ***Right Recovered G-Ratio:*** Histogram shows g-ratio of all axons in formalin-fixed tissue samples taken from right sides of animals that were implanted with the NCP medical device but received no electrical stimulation (sham). Axons that were incomplete or partially contained outside the image capture were not included. n=191 axon measurements/2 animals

Table 5: Summary of G-Ratio			
	Mean	**Standard Deviation**	**Mode**
Right Recovered	0.31	0.10	0.32
Right Standard Fixation	0.33	0.09	0.32
Left Recovered	0.32	0.11	0.30
Left Standard Fixation	0.33	0.09	0.32
All Recovered (R&L)	0.32	0.10	0.32
All Standard Fixation (R&L)	0.33	0.09	0.32

Conclusion

More research must be done to conclude the safety and efficacy of the neuro-cybernetic device on myelinated nerve. However, several conclusions can be drawn from this research. First, the formalin recovery technique does allow ultra-structural detail to be viewed using TEM. This is important because it establishes a technique to perform retrospective TEM studies on formalin-fixed tissue, thereby reducing future animal studies and expense. Second, the tissues exposed to the NCP device showed an increase in mean myelin area and standard deviation when compared to control tissue samples. This may indicate damage, but more research must be done to determine the significance of this finding. Third, comparison of recovered and control tissue showed similar trends (i.e. left tissue samples showed lesser mean myelin area than right tissue samples in both recovered and control tissues). While different means in control samples and recovered samples may not allow groups to be compared directly, trends seen in control tissues are mirrored in recovered tissue. Lastly, the mean g-ratio in exposed tissue samples was consistent with the control tissues. This may indicate that myelinated nerves are structurally and

functionally sound, but more research must be done to determine the significance this finding. Overall, this was a preliminary study that provided a basis of information for further studies.

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