

NERVE REGENERATION INDUCED BY
COLLAGEN-GAG MATRIX IN COLLAGEN TUBES

by

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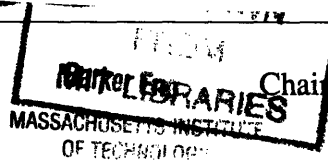
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Aria Landstrom

Submitted to the Department of Mechanical Engineering on August 23, 1994 in partial fulfillment of the requirements for the degree of Master of Science

ABSTRACT

Approximately 200,000 nerve repair surgeries are attempted every year. Unfortunately, the long-term outcomes of these repairs are far below the original functioning of the nerve. Previous studies have shown that bridging a gap in the sciatic nerve of the rat with a silicone tube filled with a porous matrix of collagen and chondroitin-6-sulfate greatly enhanced regeneration compared to a saline-filled silicone tube. In clinical use the silicone tubes present problems for long term recovery because they become encapsulated over time as part of a foreign-body response that constricts the nerve and necessitates a second surgery to remove the tube. In this study a nerve prosthesis consisting of a porous collagen-chondroitin-6-sulfate matrix ensheathed in a biodegradable collagen tube was developed as a potential method of treatment not requiring a second surgery. Two types of tubes were tested, porous collagen and non-porous collagen, both filled with the matrix. These prostheses and controls of silicone tubes with matrix were implanted in the sciatic nerves of rats. The ensuing regeneration was assessed after six weeks using NIH Image for image analysis of regenerated axons and qualitative histological examination. The results showed that the two collagen tubes performed approximately comparably with the silicone tube. In addition, no adverse reactions to the tubes were observed.

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Title: Professor of Polymer Science and Engineering

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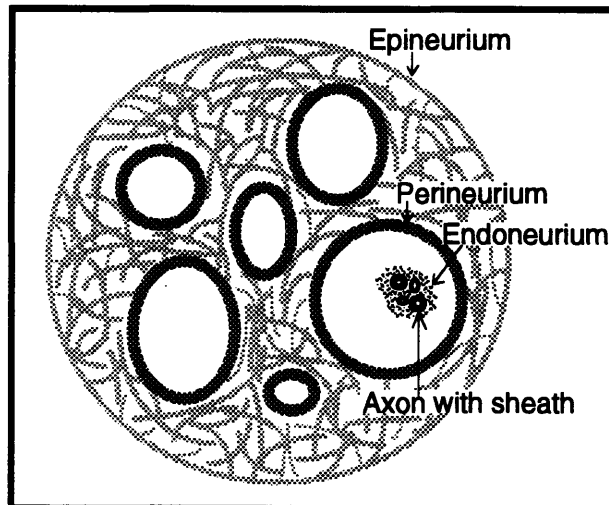
1 INTRODUCTION

1.1 PERIPHERAL NERVE INJURIES

There are many types of injuries that can result in peripheral nerve damage, including lacerations, puncture wounds and gun shot wounds. Destruction of the fibers enervating a limb generally results in amputation due to complications such as infection and further injury that can result from paralysis. In an effort to restore function to damaged nerves, approximately 200,000 nerve repair surgeries are attempted every year (Madison, 1992). Unfortunately however, the long-term outcomes of these repairs are far below the original functioning of the nerve. Many factors can adversely affect the regeneration of a nerve: sprouting axons may be blocked by scar tissue, the axons may form a painful and non-functional mass known as a neuroma, the nerve fibers may reconnect to the wrong end organs, and an end organ muscle may be atrophied beyond recovery by the time it is reinnervated.

A peripheral nerve is composed of many elements. The basic starting block is the axon, a long cellular process that reaches from the cell body near the spinal cord to the sensory or motor target. Each axon is ensheathed by Schwann cells and surrounded by endoneurial tissue. The axon may be wrapped many times in the membrane of the Schwann cell, in which case it is referred to as myelinated for the myelin that makes up the

Figure 1-1: Schematic diagram showing structure of a peripheral nerve (not to scale). The dark gray circles represent fascicles, which contain axons, Schwann cells, endoneurium, fibroblasts and capillaries.



membrane, or it may simply invaginate once into the Schwann cell, and is unmyelinated. Groups of axons are bundled into fascicles tightly surrounded by the perineurium, and the fascicles in turn are grouped by the epineurium (see Figure 1-1) (Sunderland, 1990). In humans, about one-third of the axons are myelinated, which means that the Schwann cells wrap themselves tightly around the axons, creating a layer of membrane of myelin around the axon.

Nerve injuries are clinically classified by which structures of the nerve are damaged. The most severe injury is transection of the whole nerve, including the epineurium, the perineurium, the endoneurium, the sheaths, and all axons. The response of the nerve to transection has been detailed by many researchers (Sunderland, 1990, Fawcett and Keynes, 1990, and Seckel, 1990). Following axotomy, the distal end of the cut nerve immediately begins to undergo Wallerian degeneration. In this process, the Schwann cells dissociate from the severed axons and macrophages move in to phagocytose the dead axonal material and excess myelin, with help from the Schwann cells. This process releases neuronotrophic factors and stimulates the proliferation and migration of the Schwann cells towards the proximal nerve stump. The proximal nerve stump, meanwhile, undergoes traumatic degeneration, a sequence similar to Wallerian degeneration.. This process begins from the site of transection and progresses towards the cell body at least back to the next node of Ranvier. In the worst case, traumatic degeneration results in the death of the cell. If the cell survives, the axon may begin to sprout within a day, starting from the first node of Ranvier proximal to the traumatic degeneration.(Archibald, Lisney) Regeneration of the nerve may be defined as axons traversing the injury and reconnecting with appropriate end-organs. Full regeneration of a nerve has never been achieved, only varying degrees of repair in which some or all of the axons fail to reconnect due to lack of direction and/or blockage by scar tissue.

One very effective means of studying the regeneration of severed nerves is to create a wound chamber by entubating the stumps with a gap in between them. Using this model, researchers can isolate the nerve ends from the surrounding environment and easily identify and remove the regenerated tissue. The response has been well quantified for the model of a 10 mm gap in the sciatic nerve of the rat entubated in silicone rubber (Williams, et. al., 1983, Fields, et. al., 1989) Research has shown that under these conditions, the tube fills with fluid exudate from the stumps within the first day, and a fibrin matrix is formed spanning the gap within the first week. In the second week, a tissue bridge of Schwann cells, perineurial cells and endothelial cells is formed which acts as a guide for the axons. The axons make their way across the gap in approximately four weeks and proceed through the distal stump towards end organs via empty endoneurial tubes. Myelination of a regenerated portion of axon generally lags by at least five to eight days. Since there may be several sprouts from each axon, the axon count may be higher in the regenerated portion of the nerve than in the intact portion proximal to the injury. Theoretically, over the long term only the sprouts that successfully reach a target survive and mature fully, while those that fail to make a connection die out.(Fawcett and Keynes, 1990)

1.2 METHODS OF PROMOTING REGENERATION

The primary focus of experimental methods attempting to promote and improve peripheral nerve regeneration has been guidance of the axonal growth cone to the distal stump. These efforts center around entubating the proximal and distal nerve ends with various materials. Entubation is believed to aid regeneration by preventing displacement of the nerve stumps, protecting the regenerating tissue, orienting axonal growth and vascularization, reducing invasion by connective tissue that causes scarring, and concentrating growth and trophic factors (Fields, et.al., 1989). Silicone entubation was the standard experimental model for many years and increased regeneration compared to

non-entubated controls. However, in clinical use the silicone tubes presented problems for long term recovery. The silicone became encapsulated over time as part of a foreign-body response, which constricted the nerve and necessitated a second surgery to remove the tube. To avoid this problem, the trend has been towards biological and biodegradable materials. Autogenous grafts of pseudosynovial sheaths, veins and other materials are being investigated (Lundborg, et.al., 1979, Mackinnon, et.al., 1985, Chiu, et.al., 1982, Heijke, et.al, 1993). Non-tissue biodegradable tube materials have included polyglactin, biodegradable polyurethane, poly-L-lactide and poly- ϵ -caprolactone and collagen (Molander, et. al., 1983, Robinson, et. al., 1991, den Dunnen, et. al., 1993a&b, Archibald, et.al., 1991, Rosen, et.al., 1990). Overall, the qualities that are considered to be most important for a nerve guide tube are: 1) biodegradable without releasing toxic, antigenic or carcinogenic substances, 2) thin and flexible, 3) inhibitory to adhesions, fibrosis and other pathologies, 4) controllable resorption rate to be compatible with rates of axon growth and maturation, and 5) to promote vascularization of the regenerated nerve and accumulation of growth promoting factors (Madison, et.al., 1992, Fields, 1989).

In addition to gross entubation for the nerve, some researchers believe that a substance directly in the gap between nerve ends may improve regeneration. Such a substrate could potentially provide more specific directional orientation for the axons, enable haptotaxis, and contain growth and trophic factors (either exogenous or absorbed *in vivo*). Efforts in this direction have included laminin and collagen gels, fibronectin clots and skeletal muscle basal lamina grafts (Valentine, et.al., 1987, Bryan, et.al., 1993, Glasby, et.al., 1986). The results of these efforts have been mixed: while some substances did improve regeneration, others actually impeded it. Previous research in this lab indicated that a matrix of collagen and chondroitin-6-sulfate (a glycosaminoglycan) inside a silicone tube greatly enhanced regeneration compared to a saline-filled tube implanted *in vivo* in a gap in the sciatic nerve of rats.(Orgill, 1987, Chang, 1988).

1.3 GOALS OF PROJECT

The goal of this project was to develop a nerve prosthesis including the collagen-glycosaminoglycan matrix that could be used clinically in humans by using a biodegradable collagen tube instead of the silicone tube used in previous studies. In order to accomplish this, the manufacturing and processing procedures were modified for the use of collagen tubes. Prostheses of silicone, porous collagen and non-porous collagen with and without collagen-glycosaminoglycan matrix were then implanted in the sciatic nerve of rats and the regeneration was assessed histologically after six weeks. Comparison was made to normal, intact nerves and to nerves regenerated after a simulated autograft operation performed by resecting a 10 mm section of nerve and suturing it back in place.

2 MATERIALS AND METHODS

2.1 PRODUCTION OF COLLAGEN-GAG SLURRY

The preparation of the collagen-GAG (CG) matrix started with preparation of an aqueous dispersion. Fibrous freeze-dried bovine hide collagen was first ground in a Wiley Mill (Arthur J. Thomas Co., Philadelphia, PA) with a 20 mesh screen and liquid nitrogen cooling. 1.65 g of the ground collagen was then blended with 600 ml of 0.05 M acetic acid (glacial acetic acid, Mallinckrodt Chemical Co., Paris, KY) for 1 hour at 4° C on the HIGH speed setting (Granco overhead blender, Granco Co., Kansas City, MO; Brinkman cooler model RC-2T, Brinkman Co., Westbury, NY). After the hour, 120 ml of 11% w/v chondroitin-6-sulfate (from shark cartilage: no. C-4384, Sigma Chemical Co., St. Louis, MO) in acetic acid 0.05 M was added dropwise to the blending collagen dispersion over 15 minutes using a peristaltic pump (Manostat Cassette Pump, Catalog no. 75-500-0.00, Manostat, NY, NY). The mixture was blended 15 minutes longer, then centrifuged at 2300 rpm for 1 hr at 4° C (Damon Centrifuge model CRU-5000, Damon International Equipment Co., Needham Heights, MA). 420 ml clear supernatant was decanted, and the precipitate was reblended on the LOW speed setting for 15 min. at 4° C.

2.2 PROSTHESIS MANUFACTURING AND PREPARATION

The extra-cellular matrix (ECM) analog for the nerve prostheses was prepared from the CG slurry by deaerating it under vacuum at 30 mmHg for 10 min. with agitation, injecting it into silicone tubes (Dow-Corning model 602-235 medical grade Silastic 0.058" ID, 0.077" OD, Dow-Corning Co., Midland, MI) plugged with silicone adhesive (Medical Adhesive Silicone Type A, Dow-Corning Co., Midland, MI). The filled tubes were then inserted into PVC (0.125" ID, 0.25" OD) jackets and lowered at 10^{-4} m/s into a cooling bath at -80° C (Loree, 1988). This set of conditions was chosen to yield axially-oriented pores in the collagen matrix with an average diameter of 5 μ m, which yielded the most promising results in previous studies (Chang, 1988, Perutz). After freezing, the filled

tubes were removed from their jackets, the plugs were cut off, and the tubes were freeze dried for 12 hours at 100 mTorr (FTS FreezeDryer serial no. SD-8-87-16, FTS Systems, Inc., Stone Ridge, NY) to sublimate the water out of the frozen slurry. The result after freeze drying was a delicate, sponge-like matrix of collagen and GAG. The tubes were placed in open foil envelopes and de-hydro thermal (DHT) treated for 24 hrs. at 105° C and 30 mmHg (Fisher Isotemp vacuum oven, Fisher Scientific, Medford, MA) to crosslink the collagen and sterilize the matrix. The envelopes were sealed upon removal from the oven and the contents handled with sterile techniques.

Three types of tubes were used for prostheses in this study: silicone (Dow-Corning model 602-235 medical grade Silastic 0.058" ID, 0.077" OD, Dow-Corning Co., Midland, MI), porous collagen (Collagen Nerve Conduit, 1.5 mm ID, 1.60 mm OD, crosslinked 1 hour 37% formaldehyde, Integra Life Sciences, Inc., Plainsboro, NJ), and nonporous collagen (Collagen Nerve Conduit, 1.5 mm ID, 1.56 mm OD, non-crosslinked, Integra Life Sciences, Inc., Plainsboro, NJ). The tubes were sterilized in the vacuum oven 24 hrs. at 105° C and 30 mmHg, the same as the matrix.

The prostheses were assembled a few days before surgery by trimming the tubes to 20 mm and inserting a 10 mm length of matrix carefully extracted from the silicone tubes into the center. These were then stored in specimen jars filled with sterile PBS until implantation. Six types of prostheses were made: empty silicone tubes (designated Se), silicone with matrix (Sm), empty porous collagen (PCe), porous collagen with matrix (PCm), empty non-porous collagen (NPCe), and non-porous collagen with matrix (NPCm).

2.3 SURGICAL IMPLANTATION AND SACRIFICE

Adult female Sprague-Dawley rats weighing 230-250 g were anesthetized with an intraperitoneal injection of sodium pentobarbital, 50 mg/kg. After positioning rat prone with its back legs in 30° abduction, a 4 cm incision was made on the posterolateral side

and the sciatic nerve was bluntly dissected through the lateral intramuscular septum (see Figure 2-1). The nerve was further anesthetized by dripping several drops of Lidocaine over its exposed length before transecting midway between the sciatic notch and the point where the nerve branches near the knee. The nerve ends were inserted 5 mm into the prosthesis and sutured in place with two opposing 10-0 nylon sutures, as shown in Figure 2-2. For the autograft group, a 10 mm portion of the nerve was resected and sutured back into place with three 10-0 nylon sutures. The muscle was closed with three 4-0 sutures, and the skin with five 3-0 sutures covered by 3 mm staples (the rats quickly removed skin closures, so the redundant system slowed them down and gave the wound more time to heal). During the six-week recovery period, the rats were in cages with paper pellet linings and given food and water ad libitum. The rats were examined weekly to observe the recovery and check for signs of autotomy.

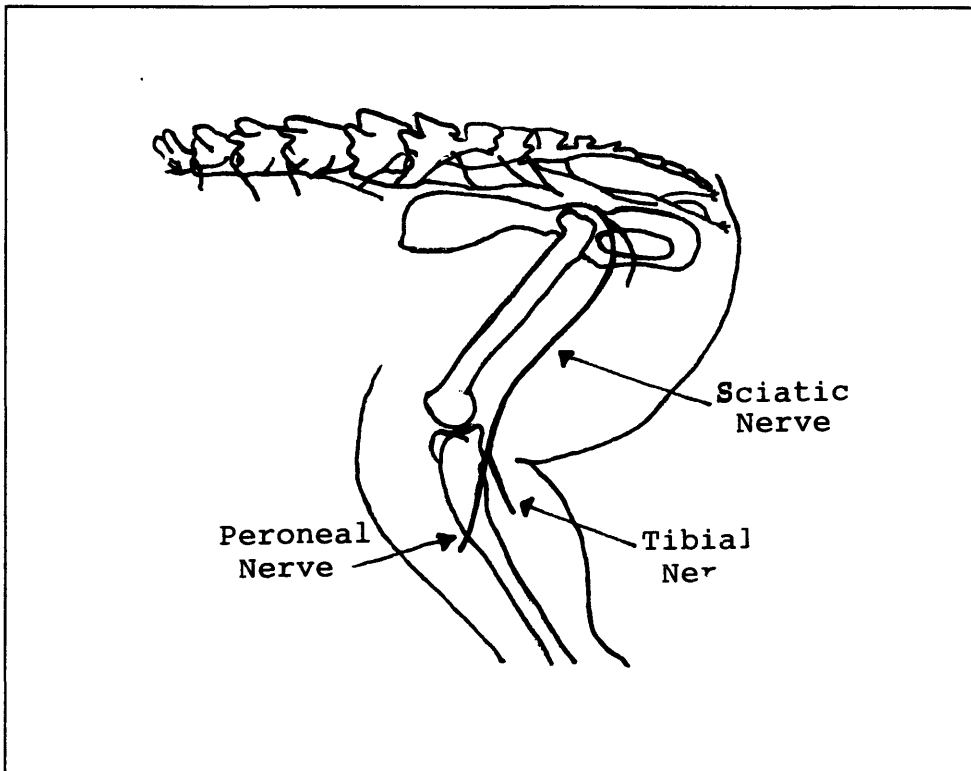


Figure 2-1: Rat hindquarter, showing location of sciatic nerve.

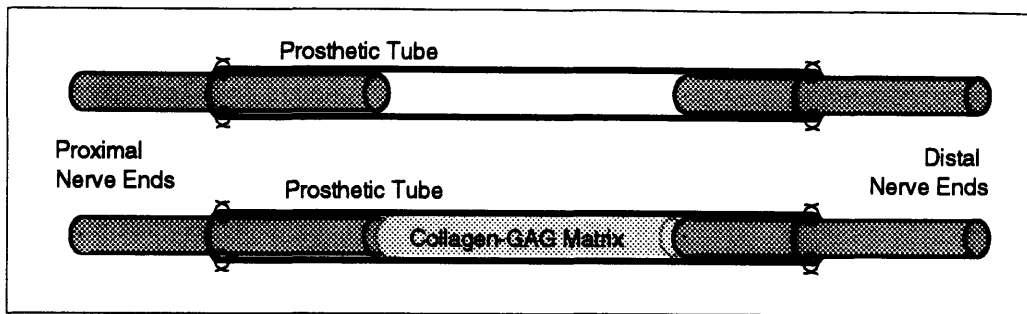


Figure 2-2: Arrangement of prosthesis and nerve, the top figure shows an empty tube and the bottom includes the CG matrix..

At the time of sacrifice, the animals were re-anesthetized with 50 mg/kg pentobarbital, as for surgery. The chest was opened and the heart exposed to allow insertion of an 18 G cannula into the left cardiac ventricle. The right auricle was cut open to allow drainage. The animal was first perfused with 15 ml Heparinized saline (20 units per milliliter), then with 300 ml cold perfusate consisting of a 1:1 mixture of Yanoff's solutions A and B (see Appendix A.5 for perfusate ingredients). After fixation, the nerves were explanted from the sciatic notch to the knee. The explanted nerve was photographed and placed in a labeled specimen jar with the perfusate solution.

2.4 HISTOLOGICAL PREPARATION AND ANALYSIS

The excised nerves were postfixed in the perfusate solution for 24 hours at 4° C. Several means of examining the nerves were desired. Table 2-1 shows the grid of histological features studied and how each was analyzed. In order to view the desired features, the explanted nerves were sectioned as shown in Figure 2-3.

Table 2-1: Histology grid

Feature of Interest	Site	Fixation	Embedding Medium	Stain
Axons	E3, E7, E10	Perfusion, protocol, osmium	Epon	Osmium
Connective Tissue	P2, P5, P8, P11	Perfusion, formalin	Paraffin	Masson's Trichrome
Gross Appearance	P2, P5, P8, P11	Perfusion, formalin	Paraffin	Hemotoxylin & Eosin

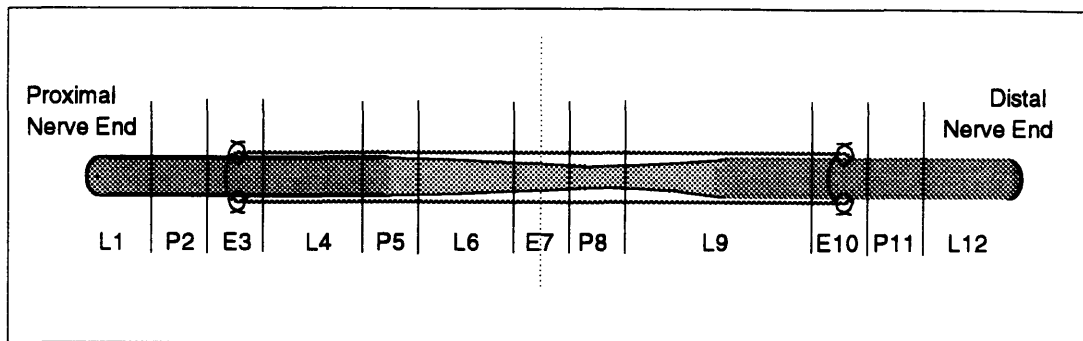


Figure 2-3: Sectioning of explanted nerve for histology. Dark gray shows the intact tissue, while the light gray shows regenerated tissue and nerve. The letters in the section name stand for the method of processing: L = liquid storage, P = paraffin embedding, E = epon embedding.

The theoretical distance between sutures was 20 mm, with each of the epon and paraffin sections 2 mm in length. However, the length of the actual explanted nerves varied widely and adjustments had to be made. Before cutting, the nerves were first measured, then the sections were marked off with different color markers in order of section priority: epon, then paraffin, then liquid storage. Sections E3 and E10 were marked to straddle the sutures, and E7 was centered between the sutures. The position of the paraffin sections was referenced to the epon sections. After marking, the nerve was cut with a fresh #11 blade and each section was placed in a labeled 1 dram vial filled with Neutral Buffered Formalin.

After recording the unavailable sections, the nerves were processed according to media. The liquid storage sections were left in formalin for 48 hours, then transferred to 70% ethanol and stored. After 48 hours in formalin, the paraffin sections were sections were dehydrated in graded alcohol, cleared in xylene, and embedded in paraffin. 3-5 μm thick sections were cut on a microtome, mounted on slides, and then stained (each separate) with Masson's Trichrome (Trichrome) and Hemotoxylin & Eosin (H & E). Masson's Trichrome is used to identify connective tissue, it stains native collagen blue-green and stains cells red. The Trichrome slides were examined and evaluated for the quantity of intrafascicular collagen and the thickness of epineurial tissue. Hemotoxylin

and Eosin is the standard stain for evaluating cells: nuclei are stained purple and cytoplasm is stained pink. The sections were examined for the presence of acute inflammatory cells (AIC's) such as macrophages, lymphocytes and monocytes that could indicate an adverse cellular reaction to the prostheses.

The epon sections were transferred from the formalin after 24 hours to 2% cacodylate-buffered glutaraldehyde (pH 7.4), and soaked for 30 hours at 4° C. They were then post-fixed in 1% buffered osmium tetroxide, dehydrated in graded alcohol, cleared in acetone and embedded in epon. 1- μ m thick sections were cut on an Ultratome. If the osmium did not produce enough contrast to make the axons readily visible, the sections were further stained with Nile Blue Hydrochloride. After carefully examining the sections, a representative portion was digitized via a microscope with a video camera and the program DIGIT (Sept. 26, 1993 version) to capture the image. The image analysis was performed on a Power Macintosh 6100/60 using the public domain NIH Image program (written by Wayne Rasband at the US National Institutes of Health and available from the Internet by anonymous ftp from [zippy.nimh.gov](ftp://zippy.nimh.gov) or on floppy disk from NTIS, 5285 Port Royal Road, Springfield, VA 22161, part number PB 93-504868). Each axon in the image was traced in the "Density Slice" mode, and "Analyze Particles" was used to measure the size of each axon. This data was pasted into spreadsheet files, where size histograms of the axons were generated and compared.

3 RESULTS

3.1 GROSS APPEARANCE

Thirty-five rats were initially used in this study, five for each of the six prosthetic groups (silicone empty (Se), silicone with matrix (Sm), non-porous collagen empty (NPCe), non-porous collagen with matrix (NPCm), porous collagen empty (PCe), and porous collagen with matrix (PCm)), and five for the autografts (AG). The initial number of five was chosen to yield three competent specimens from each group (based on survival for the full time period and successful retrieval of the nerve). If more than three nerve samples were available for a given group, three were randomly selected for examination. Five rats had to be sacrificed before the designated six week time period, and one died of unknown causes. Two rats were added to replace ones that died very early, for a total of thirty-seven operations. Each rat was operated on on one leg, and the normal, intact sciatic nerve (Norm) from the unoperated leg was also extracted at the time of sacrifice from seven of the rats.

This thesis will discuss the detailed analysis of four of the seven groups: Sm, PCm, NPCm, and Norm. The gross appearance of nerves was surveyed subsequent to sectioning. Tissue spanned the surgical gap in all of the twenty-one nerves except one Se and one Sm (see Figure 3-1).

3.2 GENERAL HISTOLOGICAL APPEARANCE

3.2.1 Masson's Trichrome

Figures 3-2 through 3-5 show representative sections of each group for the Trichrome stain (all taken from section P8, distal to the center of the surgical gap). The Normal nerve shows very little intrafascicular collagen, a thin, dark blue epineurium, and some surrounding connective tissue. All of the regenerated nerve sections show an increase in intrafascicular collagen over the normal nerve by several factors. This additional connective tissue shows up as pale to dark blue in the photographs. One unexpected

finding was that the Porous Collagen tubes stained red, rather than blue (see Figure 3-4). This may have been due to glutaraldehyde cross-linking of the tubes (performed by the manufacturer to give the tubes additional strength). Overall, there was too much section-to-section variability in the quantity of intrafascicular collagen to observe any trends between groups. Very few of the regenerated nerves had an epineurium resembling the thin, fibrous layer around normal nerve. The regenerated nerves tended to have a predominantly cellular layer surrounding them that was examined through the H & E sections.

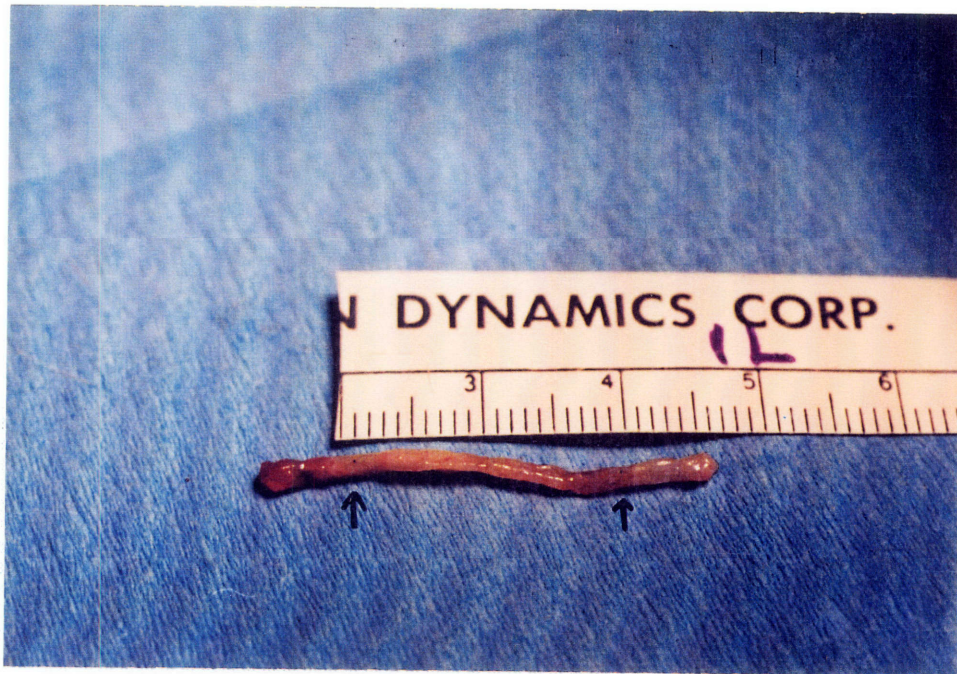


Figure 3-1: Photograph of an explanted nerve regenerated through an NPCm. Arrows point to the sutures at the ends of the tube.

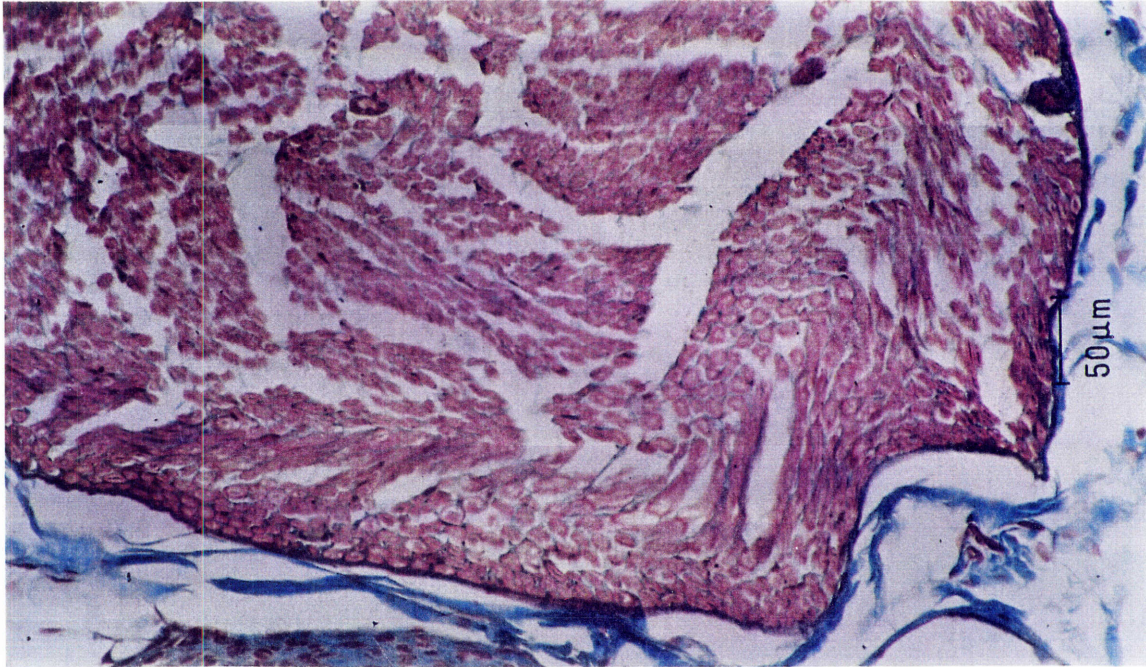


Figure 3-2: Normal, intact nerve stained with Masson's Trichrome.

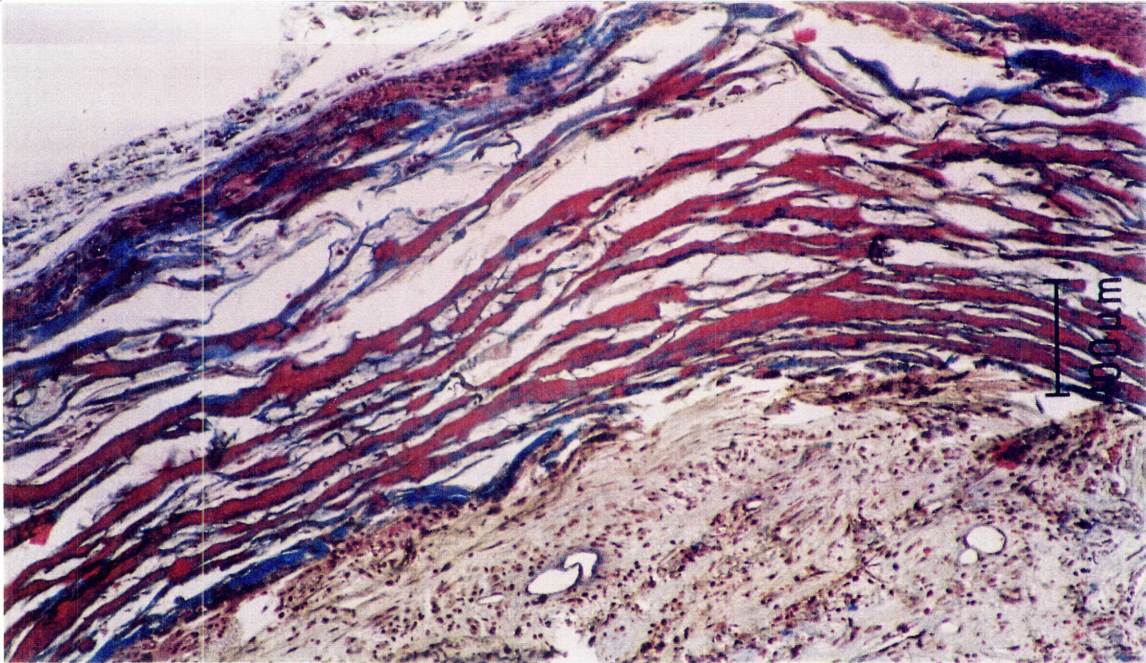


Figure 3-3: Regenerated nerve, Porous Collagen with matrix prosthesis, stained with Trichrome.

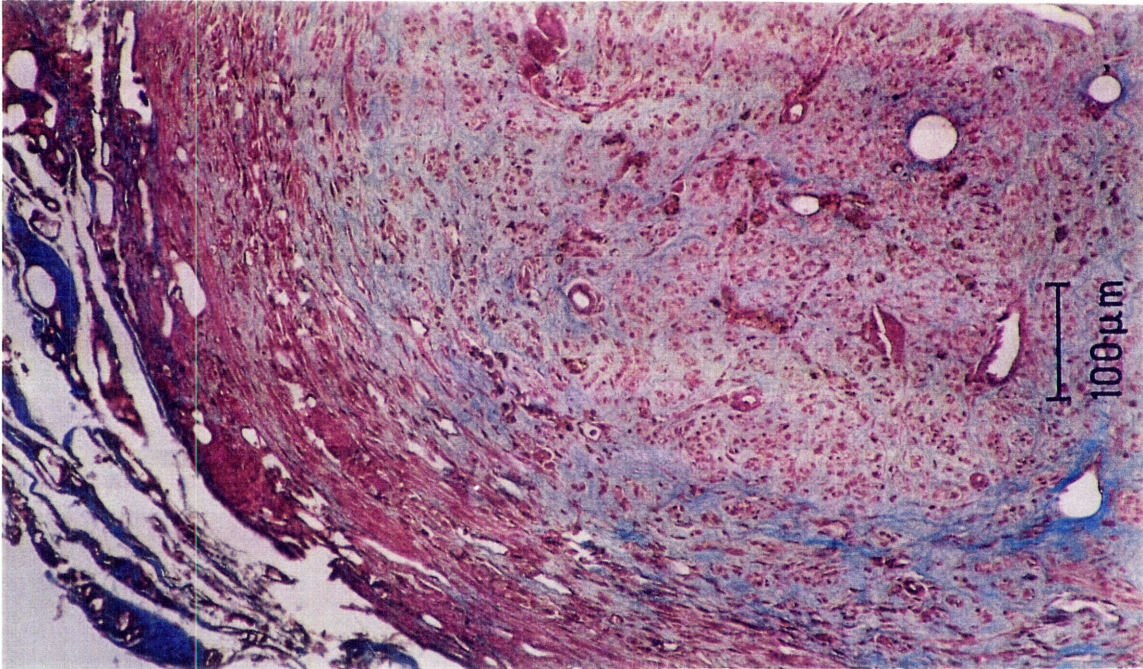


Figure 3-4: Regenerated nerve, Non-Porous Collagen with matrix prosthesis, stained with Trichrome.

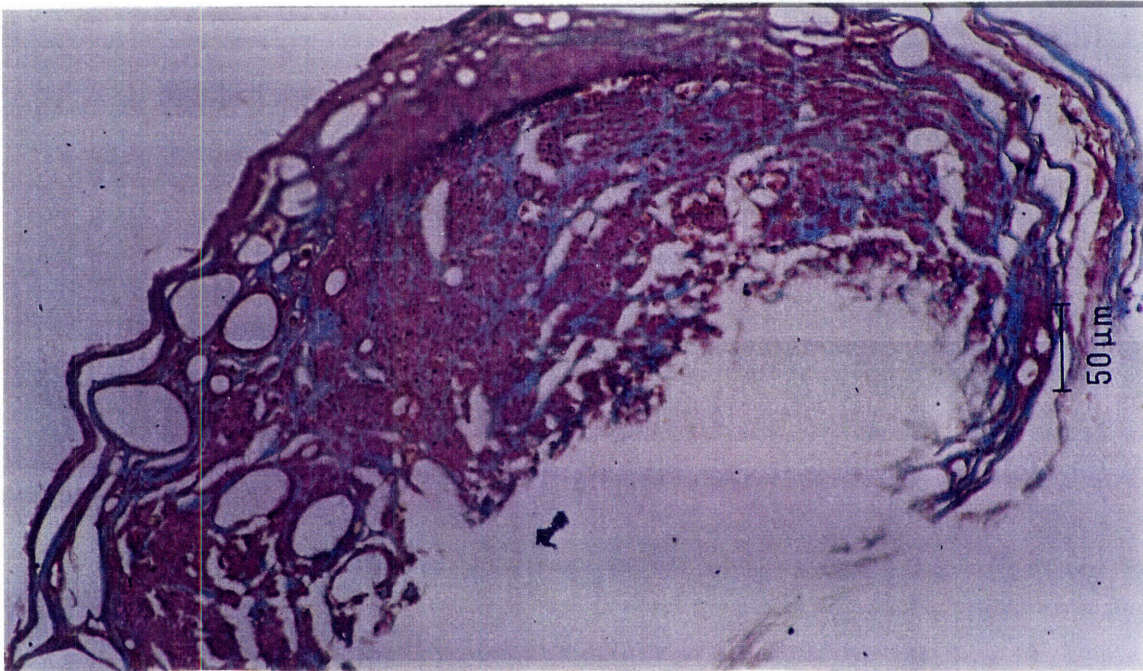


Figure 3-5: Regenerated nerve, Silicone with matrix prosthesis, stained with Trichrome. Tissue did not fill tube.

3.2.2 Hemotoxylin and Eosin

The Normal nerve contained nearly no AIC's, as expected. The regenerated sections did contain some AIC's, but not enough to indicate a significant problem. The

regenerated nerves also all had orange clumps of hemosiderin, a byproduct of red blood cell degradation. All of the regenerated nerves also showed very exaggerated numbers of blood vessels. As with the Trichrome sections, there was too much section to section variability to detect any trends among the regenerated groups as far as relative amounts of AIC's within the nerve. The Porous Collagen and Non-Porous collagen both had thick cellular layers around the outside and inside of the tube. On at least one of these sections, macrophages and giant cells were seen engulfing particles that appeared to be tube remnants, indicating a likelihood of cellular-participation in the breakdown of the tubes. Figures 3-6 through 3-9 show representative sections from each group, section P8 (immediately distal to the center of the gap).

3.3 QUANTITATIVE ANALYSIS

The Epon sections were examined and then a portion of the cross-section of each was digitized and analyzed for the number and size of axons visible in the image. The Normal nerve showed large, readily visible axons with thick myelin sheaths. The regenerated nerves all had smaller axons than the Normal nerve and much thinner myelin sheaths (partly due to immaturity of the axons). Figures 3-10 through 3-13 show photographs of the epon sections, all taken from section E7 (central to the surgical gap).

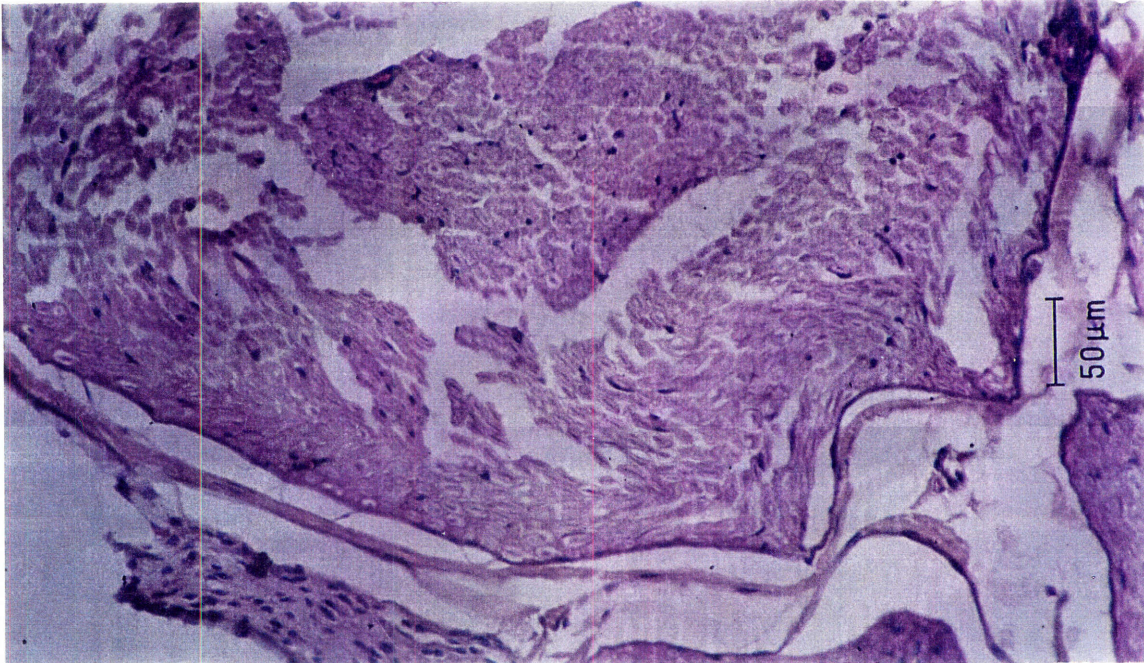


Figure 3-6: Normal, intact nerve stained with Hematoxylin & Eosin. The mature axons resemble pale purple doughnuts, few nuclei are visible within the nerve.

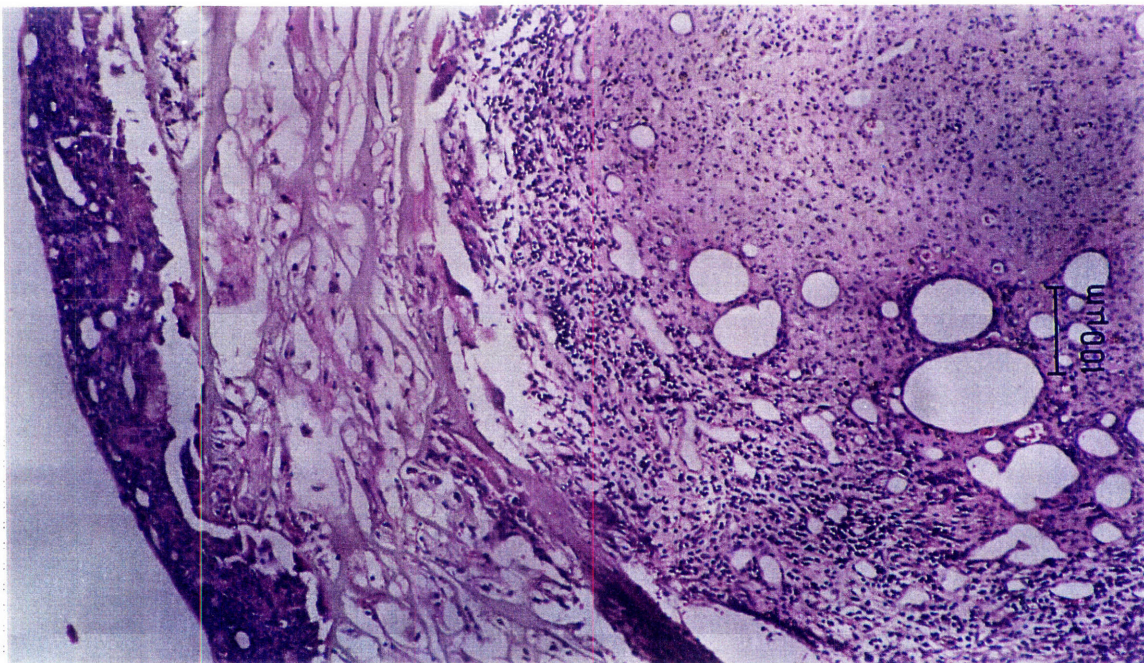


Figure 3-7: Regenerated nerve, Porous Collagen with matrix prosthesis, stained with H & E. Note the dense cellular layer surrounding the outside of the tube.

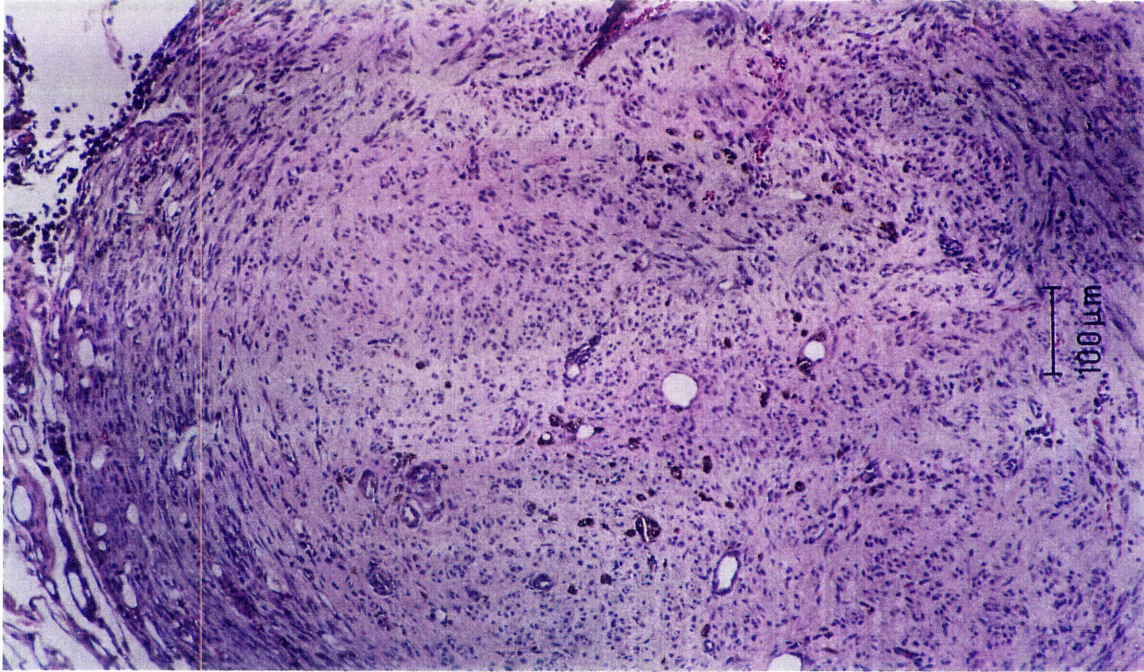


Figure 3-8: Regenerated nerve, Non-Porous Collagen with matrix prosthesis, stained with H & E. The large orange clumps are hemosiderin.

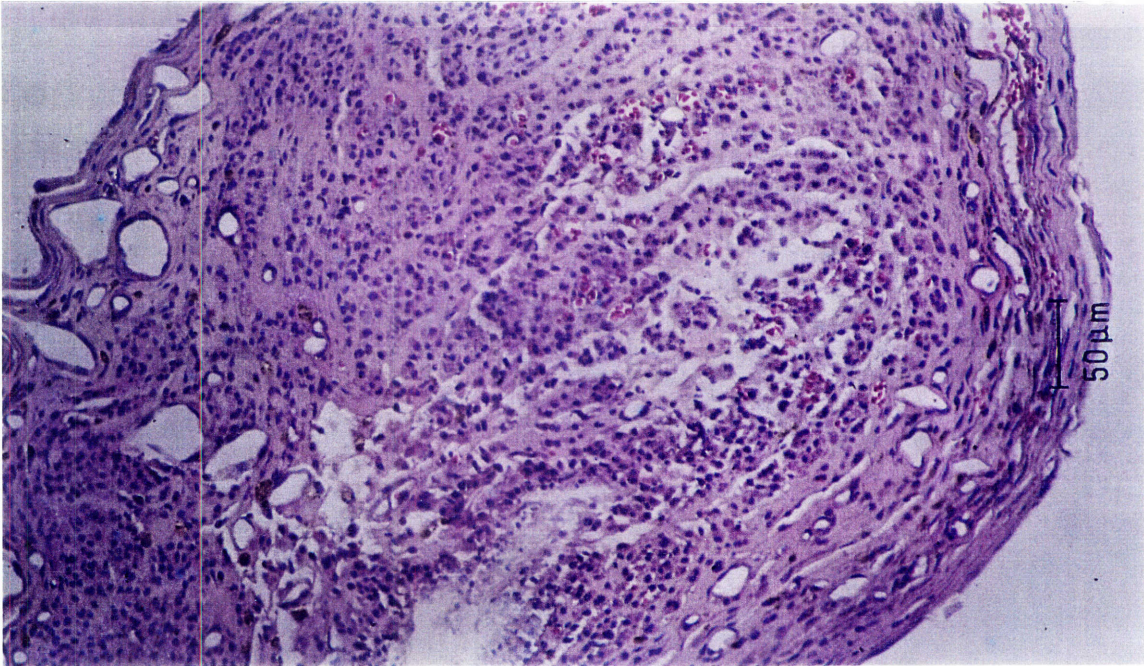


Figure 3-9: Regenerated nerve, Silicone with matrix prosthesis, stained with H & E. Note the many blood vessels, visible as open patches filled with small pink circles (red blood cells).

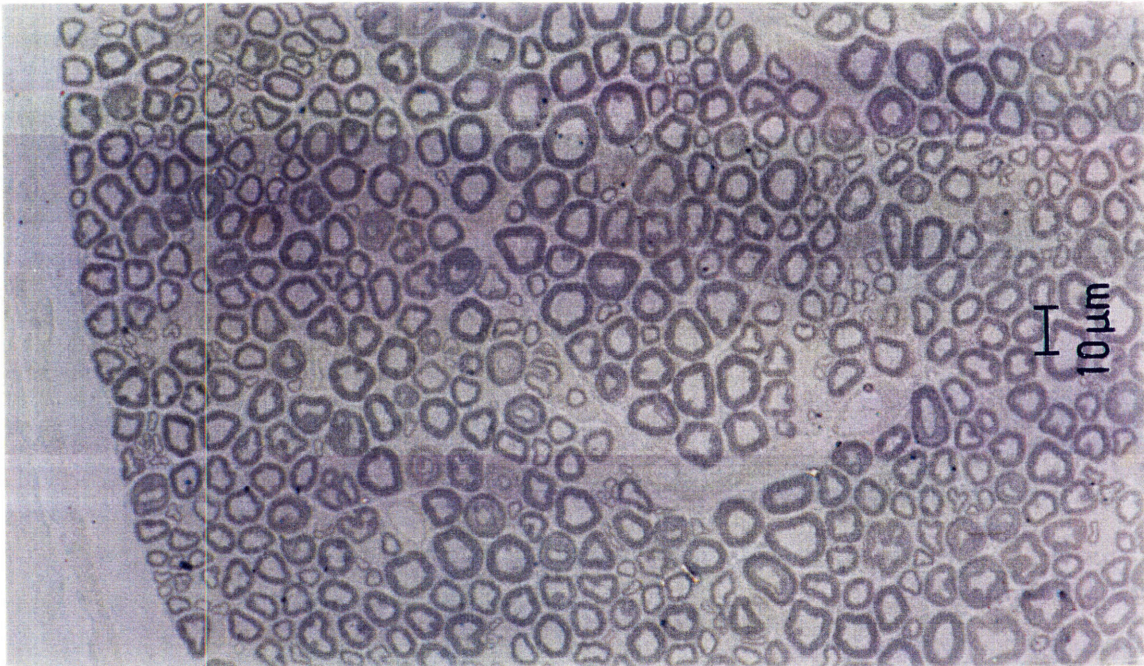


Figure 3-10: Normal, intact nerve stained with Osmium. The size distribution can be seen to be slightly bimodal, with few medium-sized axons.

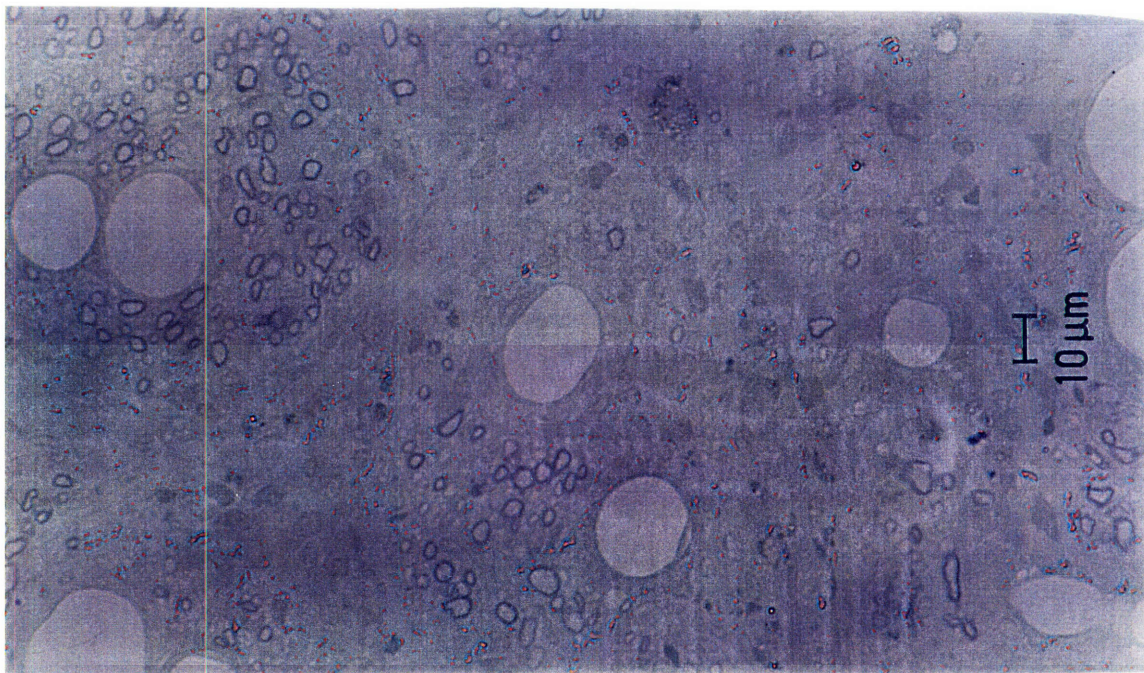


Figure 3-11: Regenerated nerve, Porous Collagen with matrix prosthesis, stained with Osmium and Nile Blue.

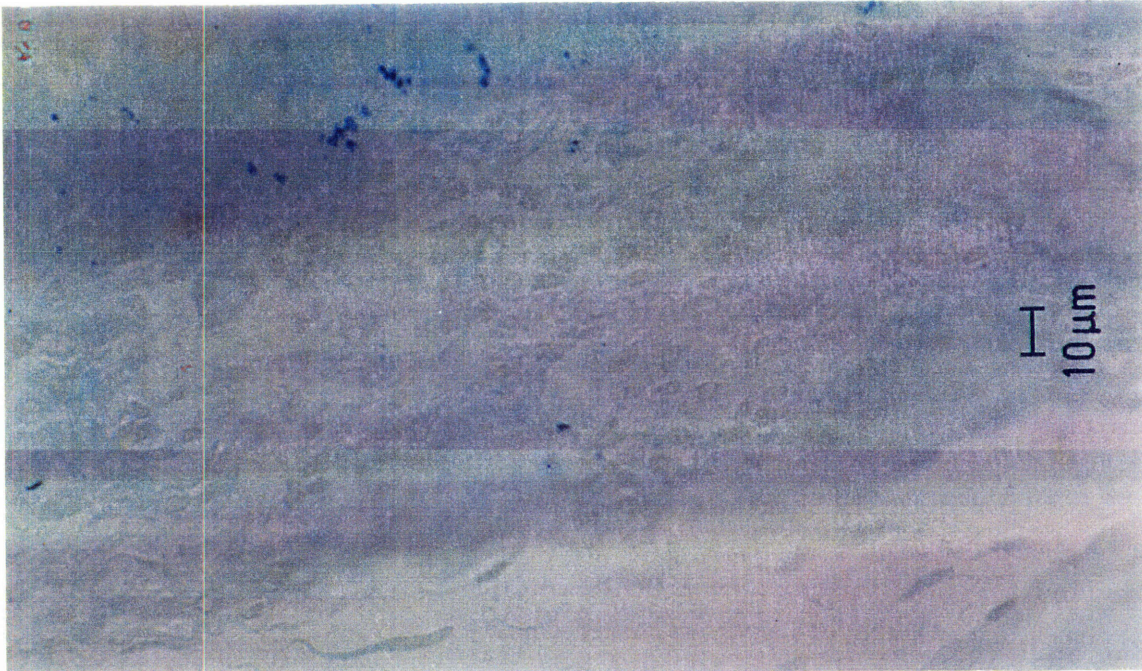


Figure 3-12: Regenerated nerve, Non-Porous Collagen with matrix prosthesis, stained with Osmium and Nile Blue. The axons are faint due to very thin myelin sheaths.

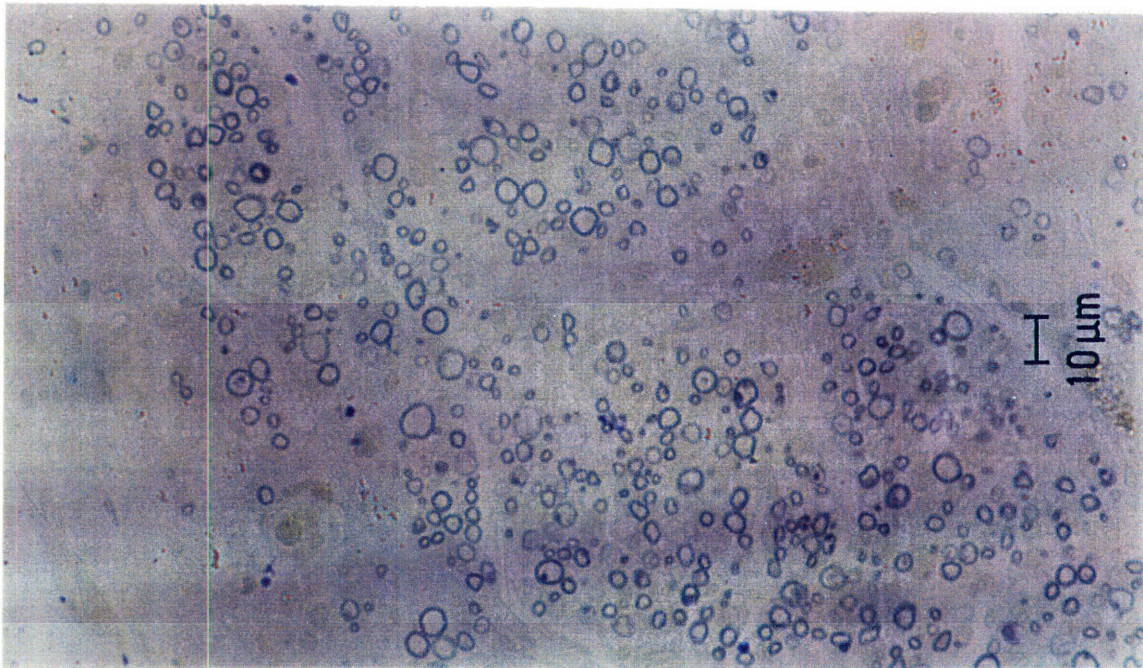


Figure 3-13: Regenerated nerve, Silicone with matrix prosthesis, stained with Osmium and Nile Blue.

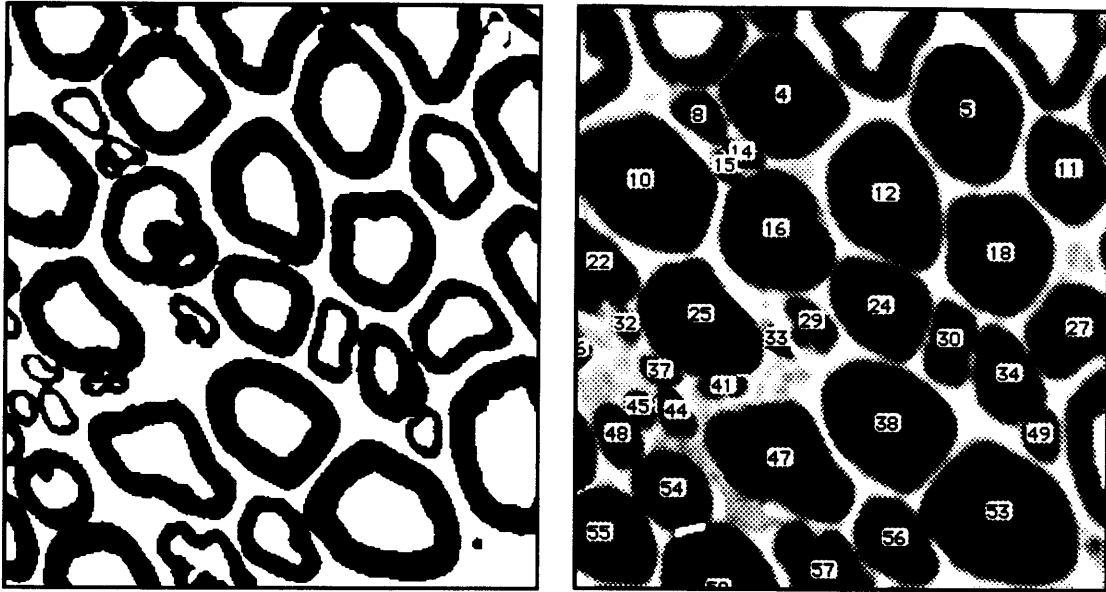


Figure 3-14: Portions of Normal nerve images, before (left) and after (right) analysis.

Image analysis as shown in Figure 3-14 was performed on each of the three epon sections (E3, E7 and E10) for each nerve. The size of each myelinated axon was measured, and then compiled into histograms. The numbers of axons in the regenerated nerves were slightly higher than the normal nerve in the section proximal to the gap, and then decreased to numbers approximately comparable to normal in the gap region and distal to it (see Figure 3-15). However, as noted from the photographs, the axons were significantly smaller than normal. The regenerated axons had a peak distribution around three μm compared to a nearly even distribution over the size range of 3 μm to 12 μm for normal nerves. Figures 3-16 through 3-18 present the exact distributions.

In the proximal region, there was no significant difference between any of the three graft groups. In both the central and distal regions, the Silicone with matrix group had more axons over much of the size distribution, but the difference is also not significant due to the high animal-to-animal variability within each group. As is shown in the graphs, the two collagen tube groups, PCm and NPCm, performed comparably.

Figure 3-15: Average total number of axons per image in each specimen group, by location.

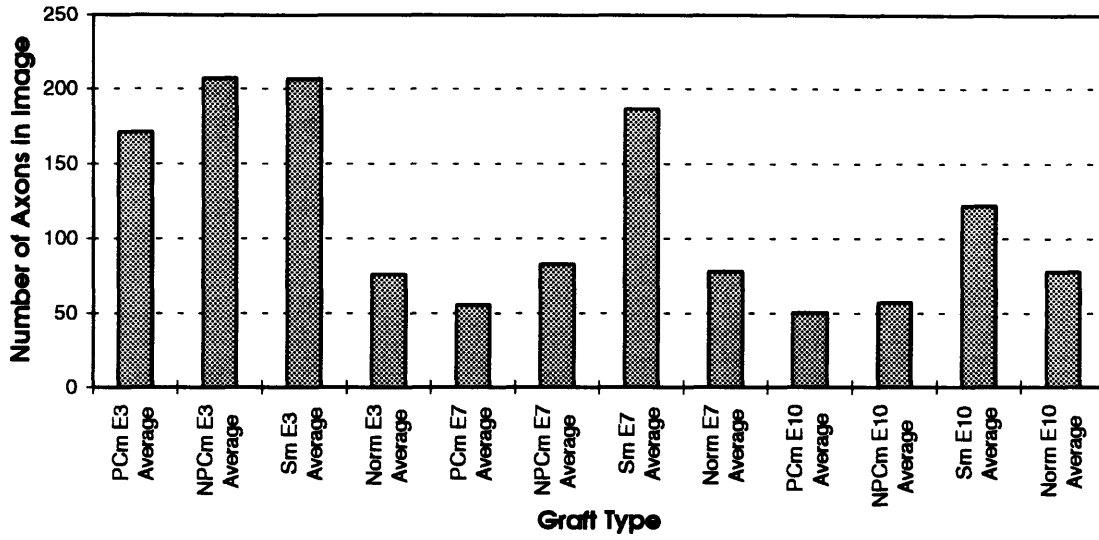


Figure 3-16: Distribution of axons in the proximal E3 section, by group.

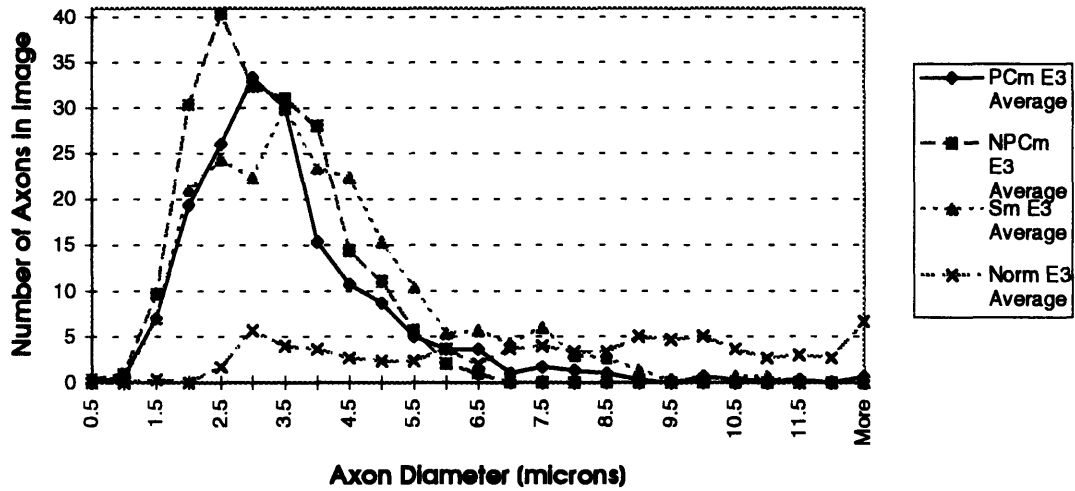


Figure 3-17: Distribution of axons in the central E7 section, by group.

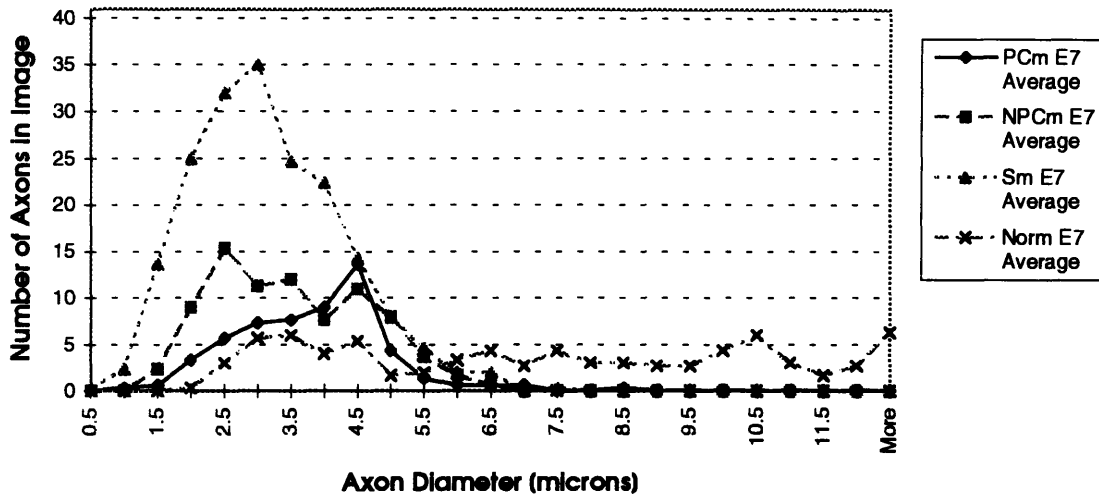
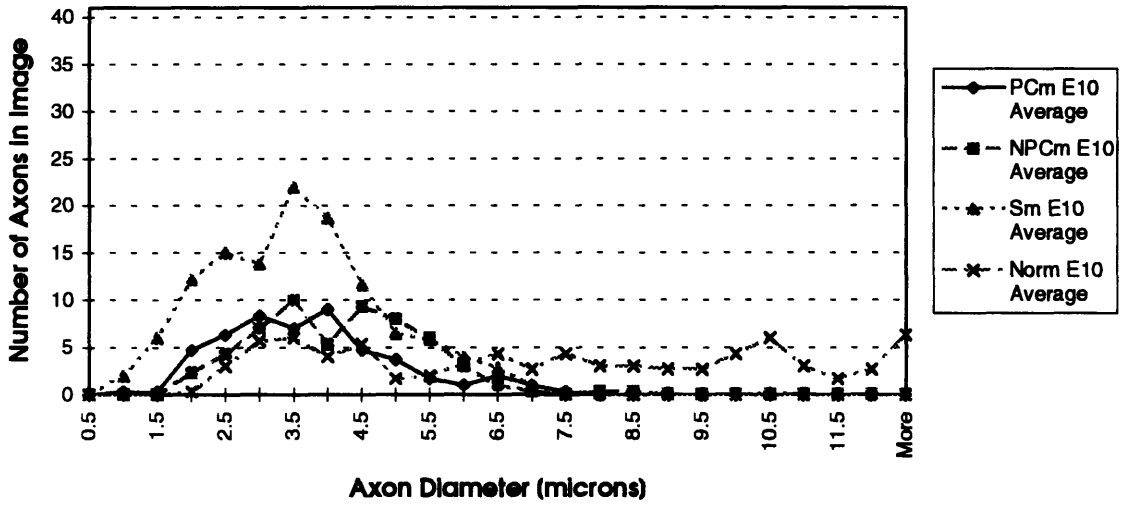


Figure 3-18: Distribution of axons in the distal E10 section, by group.



4 DISCUSSION

4.1 EFFECTIVENESS OF REGENERATION

Based on Figures 3-15 through 3-17, the matrix filled collagen tubes appear to be approximately comparable with the matrix filled silicone tubes in promoting axonal regrowth. Further analysis must be done to better determine the overall condition of the regenerated nerve and compare the different groups. The first factor that needs to be better examined is the overall regeneration. This study only quantitatively examined one small image out of the cross sectional area. Many of the nerves varied tremendously in both the quantity of tissue regenerated in the gap and the fraction of the tissue that was populated by axons. The tissue regenerated through silicone tube prostheses tended to be particularly small because the tissue did not adhere to the tube, as demonstrated by Figure 3-5.

Ideally, the image analysis would be paired with a measurement of the area of nerve tissue to obtain a projected number of axons. This was attempted, but was not accurate enough to merit reporting due to difficulties in measuring the size of the tissue. There were two major problems with making this measurement 1) many of the slides were not full cross sections as a result of the cutting procedures the Ultratome and 2) at the low magnification necessary to see the full cross-section, it was not possible to accurately differentiate nerve tissue from its surrounding connective tissue. An alternative method that was initially attempted was to make photo-mosaics of the cross sections and manually count the axons. This method was abandoned because it did not yield any size distribution information. The size distribution was judged to be critically important because the quantity of axons alone does not significantly indicate the competence of the nerve. This can readily be seen by looking at the results for the proximal section (E3), where the numbers of axons were greater than in normal nerve, but so much smaller that the regenerated nerve could not possibly function as well as normal. Given time, these

difficulties in data collection could probably be overcome by modifying the embedding and cutting protocols and developing a better measuring technique.

4.2 LONG TERM REGENERATION

While this study indicates that the prostheses allow axonal regrowth, it does not permit conclusions regarding the long term results of the regrowth. Other studies have shown that while the numbers of apparent axons become elevated during regeneration due to the sprouting of multiple axonal processes from a single nerve body, branches that don't reconnect with an end-organ die off over time. This is particularly an issue in the case of axons regenerated through silicone tubes, which undergo degenerative changes after several months of entubation (Fields, et. al., 1989). This study also did not consider the condition of the myelin sheath, which is a critical element for return of function. This could also be obtained from the raw data available if the measuring techniques in the image analysis were altered and electron microscopy was employed.

5 CONCLUSIONS

Axonal regrowth was attained through prostheses of collagen-GAG matrix in collagen tubes in this study at a rate comparable with that through matrix in silicone tubes. To more fully understand the import of this regrowth, however, future studies need to incorporate improved histological techniques, longer time periods for evaluation, and functional assessment of regeneration. Time periods ranging from six weeks to one year will yield information on the stability of the regeneration, the maturation of the axons, and the degree of functional success. The presence of axons is not the only factor necessary for return of function, the axons must also successfully reconnect with their end targets. Functional success can be measured through behavioral observations, such as measurement of walking patterns, and through electrophysiological techniques directly measuring the signals traversing the regenerated nerve.

APPENDIX A: EXPLICIT PROTOCOLS

A.1 PROTOCOL FOR COLLAGEN-GLYCOSAMINOGLYCAN SUSPENSION

1. Grind fibrous freeze-dried bovine hide collagen in a Wiley Mill (Aurthur J. Thomas Co., Philadelphia, PA) with a 20 mesh screen and liquid nitrogen cooling.
2. Turn on cooling system for blender (Granco overhead blender, Granco Co., Kansas City, MO) and allow to cool to 4° C (Brinkman cooler model RC-2T, Brinkman Co., Westbury, NY).
3. Prepare 0.05M acetic acid (HOAc) solution: add 8.7 ml HOAc (glacial acetic acid, Mallinckrodt Chemical Co., Paris, KY) to 3 liters dd-H₂O. (This solution has a shelf life of ~1 wk.)
4. Blend 1.65 g of collagen with 600ml of 0.05 M acetic acid on HIGH speed setting for 1 hr at 4° C.
5. Prepare 0.11 % w/v chondroitin-6-sulfate solution: dissolve 275 mg chondroitin-6-sulfate (from shark cartilage: no. C-4384, Sigma Chemical Co., St Louis, MO) in 250 ml HOAc using a stir bar.
6. Calibrate peristaltic pump (Manostat Cassette Pump, Catalog no. 75-500-0.00, Manostat, NY, NY) to 40 ml / 5 min.
3. Add 120 ml of chondroitin-6-sulfate solution dropwise to the blending collagen dispersion over 15 min using peristaltic pump (maintain blender at 4° C).
4. Blend 15 minutes longer at 4° C.
5. Centrifuge at 2300 rpm for 1 hr at 4° C (Damon Centrifuge model CRU-5000, Damon International Equipment Co., Needham Heights, MA).
6. Decant and discard 420 ml clear supernatant.
7. Reblend remaning solution at LOW speed setting for 15 min at 4° C.
8. Store in a capped centrifuge bottle at 4° C (will keep for up to about four months, reblend 15 min on LOW speed, 4° C before using if stored more than four weeks).

A.2 PNS NERVE PROSTHESIS MANUFACTURING STEPS

1. Deaerate CG suspension in a 1500 ml Erlenmeyer flask at -30mmHg for 10 min with agitation.
2. Prepare PVC jackets by cutting off 13 cm sections of flexible PVC tubing (.125 in ID, .25 in OD) and straightening at 105° C for 2 hrs. Seal one end of each jacket using Silastic (Medical Adhesive Silicone Type A, Dow-Corning Co., Midland, MI) and allow adhesive to cure for 24 hours.
3. Flush prosthesis tubing with deionized water (Deionizing Organic Adsorption system, Hydro Services and Supplies, Inc., Durham, NC) and cut off a 15 cm length. Tubings:
 - i.) Dow-Corning model 602-235 medical grade Silastic .058 in ID , .077 in OD, Dow-Corning Co., Midland, MI.
 - ii) Collagen Nerve Conduit, 1.5 mm ID, 1.56 mm OD for non-porous tubes, 1.60 mm OD for porous tubes, Integra Life Sciences, Inc., Plainsboro, NJ).
4. Seal the prosthesis tubes using Silastic (Medical Adhesive Silicone Type A, Dow-Corning Co., Midland, MI) by squirting a small amount of adhesive into both ends of each tube. Wipe excess adhesive and allow to cure for 24 hrs at room temperature.
5. Insert a 25 gauge needle (Becton Dickinson model 25G½, Becton Dickinson Co., Rutherford, NH) into the adhesive plug on one end of the tube. Draw 10 cc of CG suspension into a 10 cc syringe (Becton Dickinson model 5604, Becton Dickinson Co., Rutherford, NH), then attach another 25G½ needle to the syringe and expel all air bubbles. Insert the needle of the syringe into the adhesive plug on the other end of the tube and inject suspension slowly until the suspension comes out of the cup of the free needle. Remove the free needle and carefully inject more suspension until the tube is pressurized. Remove the syringe.
6. Insert each prosthesis tube into a prepared PVC jacket. Using the freezing apparatus described in Loree, 1988 freeze the jacketed tubes at a lowering velocity of 10^{-4} m/s and a bath temperature of -80° C.
7. Turn on the refrigeration and condenser of the freeze dryer (FTS FreezeDryer serial no. SD-8-87-16, FTS Systems, Inc., Stone Ridge, NY). Set the temperature to -20° C.
8. Immediately remove the prosthesis tubes from the PVC jackets and section into 25 mm lengths.
9. Place the PNS bridges on freeze-drier trays and put into freeze-drier. Seal chambers and close the vacuum outlet tube then close the seal switch and turn on the vacuum pump simultaneously. Check that the pressure drops to ~100 mTorr. Once vacuum is holding steady, turn temperature up to 0° C for 12 hrs. Break vacuum and simultaneously turn off the pump, turn off unit and remove trays.
10. Package prostheses in open aluminum foil envelopes and place in vacuum oven (Fisher Isotemp vacuum oven, Fisher Scientific, Medford, MA) for dehydrothermal (DHT)

treatment for 24 hrs at 105° C and -30 mmHg to crosslink collagen. Seal the aluminum envelopes upon removing from oven.

11. Store the envelopes with the prostheses in a dessicator chamber.

A.3 PREPARATION OF PROSTHESES FOR SURGERY

1. Turn on HEPA hood, at least 1 hr before working. Wipe down workbench with 70% ethanol.
2. Ready necessary sterile implements for work and bring to workbench:
 - 1 Teflon sheet for working area
 - 1 magnifying glass
 - 1 ring stand with clamp
 - 1 ruler
 - Forceps: 2 jewelers, 2 regular, 1 large
 - 1 surgical blade holder
 - 1 bowl for alcohol
 - 2 bottles
 - Cotton gauze pads
 - 70% isopropanol solution (make sterile using HEPA filter)
 - Phosphate Buffered Saline (PBS) solution
 - 2 #10 surgical blades
 - Envelopes with prostheses
 - Small specimen jars for prosthesis storage (one per prosthesis)
 - Whirl-pak bags (one per prosthesis)
3. Put on latex gloves, a cap, a mask, and a clean, disposable lab coat. Wipe bench with 70% ethanol.
4. Using Sterile Techniques, set up sterile field with Teflon pad. Open all tool packs and pour onto sterile field. Empty gauze into bowl and cover with sterile 70% ethanol. Fill each specimen jar with sterile PBS (if the prostheses are not going to be implanted within a week or two, store in 70% ethanol and rinse in two rinses of sterile saline solution immediately before surgery). Carefully open Whirl-pak bags and stand in hood on metal frames.
5. Put on sterile gloves and wipe off the powder with alcohol gauze pads. Set up the magnifying glass on the ring stand so that lens is approximately 6" from the tabletop.
6. Trim prosthesis tubes to 20 mm using scalpel. For silicone-tube prostheses, remove 5 mm of matrix from each end of each prosthesis using jewelers forceps and scalpel (if matrix comes out of tube reinsert it carefully). For collagen-tube prostheses, remove the matrix from a silicone tube by making two careful slits down the length of the silicone and gently pulling the matrix out with forceps. Trim off any crushed or otherwise damaged matrix, and cut remaining portion into 10 mm segments to be inserted into the center of trimmed collagen tubes.
7. Place prostheses into specimen jars with PBS, close tubes tightly, place in Whirl-paks, and close bags. Label each prosthesis by type: Se for empty silicone, Sm for silicone with matrix, PCe for empty porous collagen, PCm for porous collagen with matrix, NPCe for empty non-porous collagen, and NPCm for non-porous collagen with matrix.

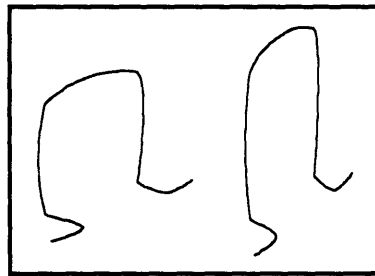
A.4 SURGICAL IMPLANTATION PROCEDURE

Animals: rats, adult female Sprague-Dawley 230-250 g (obtainable from Charles River Labs)

Hardware needed (sterile):

operating board (wood or other type of material with four pegs to tie rat down)
thumbtacks
rubber bands
surgical microscope
tongue depressor, trimmed narrow
surgical pick-ups

knife handle
#10 knife blades
jewelers forceps
tenotomy scissors
regular needle holder
microneedle holder
wound retractors made of paper clips:



Additional materials:

sodium pentobarbital (Nembutal Sodium Solution), 50 mg/ml
1 ml syringes
sterile draping
Lidocaine, 1%
nylon 10-0 sutures for prosthesis
9 mm skin staples or nylon 3-0 sutures for closing wound

Procedure:

1. Surgeon to be sterilely gowned: scrub shirt, scrub pants (optional), shoe covers, hat, mask, sterile gloves.
2. Rat is anesthetized with injection of sodium pentobarbital, 50 mg solution per kg rat. Rat should be allowed to sit 5-10 min for anesthesia to take effect. Check responsiveness to assure rat is anesthetized.
3. Operating table should be set up with tools conveniently located.
4. Rat's leg up to waist and around tail area is shaved using clippers (each rat to receive one prosthesis). Skin is sterilized with Betadine.
5. The rat is positioned prone to the board with a rolled gauze pad under the thigh and with extremities gently secured by rubber bands to thumbtacks. Lower extremities should be in 30° abduction.
6. The surgical area is sterilely draped using a towel with a 5 cm central opening.
7. A 4 cm incision is made on the posterolateral side using a #10 blade.

8. The sciatic nerve is bluntly dissected through the lateral intramuscular septum. Hemostasis is maintained.
9. Paperclip retractors are positioned transversely and horizontally. Care is taken to avoid injury to popliteal veins.
10. The nerve is anesthetized by dropping a few drops of Lidocaine onto the nerve. The nerve is positioned on the sterile tongue depressor and transected at the middle of the exposure with a fresh #10 blade.
11. The neural prosthesis is placed in the gap and proximal and distal cut nerve ends are gently inserted into both ends of the prosthesis. The nerve ends are inserted 5 mm and sutured in place using two opposing 10-0 nylon sutures, each tied with 1 double knot and 2-3 single knots. —If the distal nerve does not stay in tube, a 25G $\frac{1}{4}$ needle should be inserted into the center of the tube as a vent.
12. Skin is closed with approximately 5 surgical staples or sutures (staples are preferable because rats tend to remove sutures)
13. After implantation of prosthesis, the rats are untied and placed in cage with wood chips (wood chips are optimal for preventing autotomy, but paper pellets may be used if wood chips unavailable). Rat is observed until it shows signs of waking.
14. Rat should be checked periodically for signs of recovery or autotomy.

A.5 Animal Sacrifice Procedure --Must be carried out in hood

Equipment:

- 1 cc syringes
- 25 G needles
- sodium pentobarbital (Nembutal Sodium Solution), 50 mg/ml
- IV stand
- IV bottle for perfusate
- 1 IV bag saline, heparinized (20 units per milliliter)
- IV tubing
- 16 G cannula
- dish for perfusing
- Dissecting Kit:**
- mayo scissors
- large forceps
- small forceps
- hemostats
- knife handle
- #10 knife blades
- board for explantation
- thumbtacks
- 5-0 nylon suture
- sample containers

Yanoff's Fixative:

Solution A:

- 1.67 g Monobasic Sodium Phosphate $\text{Na}_2\text{H}_2\text{PO}_4$
- 8.95 g Dibasic Sodium Phosphate Na_2HPO_4
- 960 ml H_2O
- 40 ml Gluteraldehyde

Solution B:

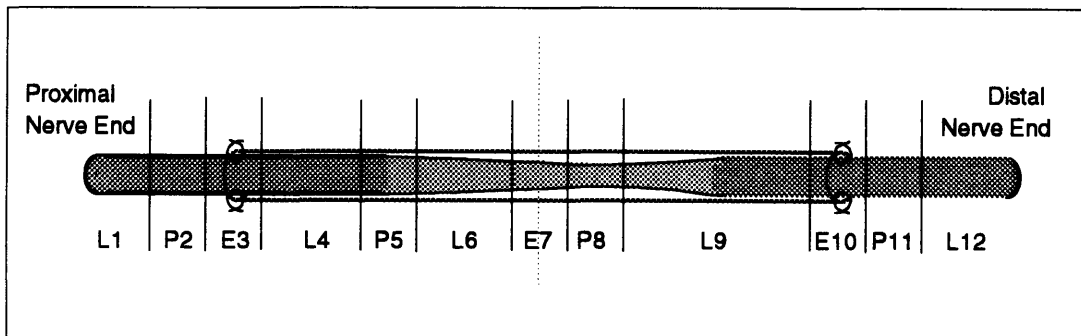
- 4.0 g Monobasic Sodium Phosphate $\text{Na}_2\text{H}_2\text{PO}_4$
- 8.95 g Dibasic Sodium Phosphate Na_2HPO_4
- 900 ml H_2O
- 100 ml 38-40% Formaldehyde

1. Anesthetize rat with pentobarbital, 50 mg solution per kg rat, as in surgical procedure.
2. Fill IV bottle with 1:1 mix of Yanoff's Solutions A and B for perfusion.
3. Set up IV tubing from perfusate and saline, hang from rack (reservoirs must be at least 100 cm above rat).
4. Place rat in dish and open chest.
5. Cut open right auricle or Vena Cava for blood drainage and position 16 gauge cannula into left cardiac ventricle.

6. Perfuse animal with 15 ml Heparized saline (20 units per milliliter) from reservoir to flush the remaining blood from the system. Follow with 300 ml ice-cold perfusate.
7. Explant both sciatic nerves, with implant tubing, from the sciatic notch to the tibial nerve branch. Clearly mark the proximal end with a suture, and place nerves in labelled sample containers filled with same perfusion solution.

A.6 HISTOLOGY

1. Post-fix excised nerves in perfusate for 24 hours with agitation.
2. Measure and record the distance between sutures (ideally, the distance should be approximately 18 mm). Mark nerve sections according to diagram below: first mark the 2 mm epon sections, which are top priority, E3 and E10 should straddle the sutures, and E7 should be centered between the sutures; then mark the 2 mm paraffin sections (all remaining material is for liquid storage).



3. Cut sections and store in labelled 1 dram vials with Neutral Buffered Formalin. Record which sections were not available. Process accordingly for each media.

Liquid Storage (L1, L4, L6, L9, L12):

After 48 hours in Solution B, transfer to 70% ethanol and store until needed.

Paraffin Embedding (P2, P5, P8, P11):

1. Dehydrate sections in graded alcohol and clear in xylene.
2. Embed nerves in paraffin.
3. Cut 3-5 μm thick sections on microtome, mount on slides.
4. Stain (each separate) with H&E, Masson's Trichrome, anti- α -actinin, and anti-neurofilament.

Epon Embedding (E3, E7, E10):

1. Fix sections in 2 % cacodylate-buffered glutaraldehyde (pH 7.4) for 30 hours at 4° C.
2. Soak nerves 24 hours in cacodylate-buffered isotonic sucrose solution (0.2 M).
3. Post-fix nerves in 1% buffered osmium tetroxide.
4. Dehydrate nerves in graded alcohol and clear in acetone.
5. Embed nerves in epon.
6. Cut 1- μm thick sections on Ultratome.
7. If axons are not readily visible, stain with Nile Blue Hydrochloride overnight.

8. Stain TEM sections with uranyl acetate and lead citrate.
9. Digitize portion of nerve at 100x (with oil), transfer image to NIH Image.
10. "Set Scale" using previously digitized images of 1 mm rule. Set Tools/Options to measure "Area" and "Perimeter/Length".
11. "Density Slice" image, "Subtract" 3, set threshold to level 254.
12. Set pen color to level 255, trace all axons visible.
13. "Analyze Particles", "Show Results". Verify that all axons traced were counted appropriately.
14. Press "Option" key and select "Copy Measurements". Paste measurements into spreadsheet.
15. Calculate the hydraulic diameter of the axons using the formula: $D = 2 * \sqrt{(Area/\pi)}$

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