Reorganization and orientation of peripheral nerve fibers regenerating through a multiple-lumen silicone rubber cuff: an experimental study using the sciatic nerve of rats

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Reorganization and orientation of peripheral nerve fibers regenerating through a multiple-lumen silicone rubber cuff: An experimental study using the sciatic nerve of rats

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Iowa State University, 1991
Reorganization and orientation of peripheral nerve fibers regenerating through a multiple-lumen silicone rubber cuff: An experimental study using the sciatic nerve of rats

by

John Mahendra Kumar Daniel

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major: Biomedical Engineering

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For the Graduate College

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Iowa State University
Ames, Iowa
1991

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1. INTRODUCTION

1.1 General

Trauma is the most common cause of peripheral nerve damage. Severe damage or severance of a peripheral nerve is caused by injuries due to violent external pressure, traction, contusion, laceration, or gunshots. The internal movements of bone fragments and jagged bone ends of a fracture can also injure a nerve. Prolonged x-ray administration, peripheral nerve tumors, intraneural injections, intraneural hemorrhage, and passage of large electric currents through the nerve tissue are some other sources of peripheral nerve damage. A number of techniques have been developed to repair damaged or avulsed peripheral nerves. The techniques chosen for repair depend on the type of injury or damage, severity of damage, and other clinical considerations. Various techniques including end-to-end suturing, fascicular suturing, nerve grafts, and nerve bridges have been used to repair human peripheral nerves. Quantitative assessment of functional recovery in nerves so repaired presents considerable difficulties. Prognostic indices of successful repair procedures are still poorly defined, and the merits of various surgical techniques remain unclear. For example, clinical assessment of functional recovery in the repaired limb is based on a subjective scale based on limb movement and reflex responses. Electromyography (which can detect muscle reinnervation) and electrophysiology have been used to quantify
the degree of recovery where feasible, but more research has to be done before these results can be used to directly compare the different repair techniques. Equally troublesome clinically are attempts to achieve nerve regeneration across a gap too great to be eliminated by surgical apposition of the proximal and distal stumps. The use of nerve autografts (a graft of tissue derived from another site in or on the body of the organism receiving it), the currently approved clinical method, is still far from perfect, and no method of bridging a nerve defect with non-biologic materials has proved clinically satisfactory. Hence there is a clear need to develop optimal peripheral nerve repair techniques for use in the clinical repair of severed or avulsed nerves.

A peripheral nerve is a complex arrangement of axons, non-neural cells, extracellular elements, and their interactions. After injury or severance of a peripheral nerve, profound morphological alterations occur in the nerve fibers and connective tissues involved in the lesion. The axons of a peripheral nerve can be transected either by severance of the whole trunk of the nerve (neurotmesis) or by blunt trauma such as crushing, which breaks the axons (axonotmesis) but leaves the non-neural supporting structures in physical continuity. The parts of the axons that have been separated from their cell bodies die and are phagocytosed by their ensheathing Schwann cells. If the cut nerve is repaired by suturing, or if the crushed nerve is simply left alone, the damaged axons in the proximal stump elongate and enter the distal stump. There, they continue to grow longer until eventually they reach and re-innervate sensory and effector organs, thereby ensuring functional recovery. It must be noted here that this sequence of events, known as axonal regeneration, is one in which cells are replacing their amputated cytoplasmic processes. It does not involve mitotic activity on the part of the neurons or the formation of new neurons from undifferentiated precursor
cells. In these respects the word regeneration has a meaning somewhat different from
the one it has when applied to cellular replacement in injured non-nervous tissue.

1.2 Statement of the problem

1.2.1 Nature of the problem

The repair of peripheral nerves is a challenging problem because of the following
reasons:

- even the most meticulous repair does not always result in normal functional
  restoration,

- it is extremely difficult for a surgeon to anticipate the outcome of the repair
  technique,

- in the clinical environment, it usually takes months, sometimes years, to prop­
erly assess the technical innovations and the results of new surgical repair re­
search methods, and finally

- there is still a great deal of information that has to be gathered about the basic
  pathophysiology of peripheral nerve repair.

Peripheral nerve repair has lagged behind other areas of reconstruction and re­
pair because of a lack of objective methods of assessing the recovery of repaired
nerves. Recovery from peripheral nerve injury can be studied by a wide variety of
techniques. Electrophysiological techniques, computerized morphometric studies and
clinical evaluation methods have been introduced to objectively evaluate the repair
techniques. Other functional tests such as the toe pinch test, toe spreading, foot-drop
estimation, flick test, and measurement of walking tracks are also used in determining functional recovery after repair. Evaluation of sensory function is imprecise because of overlapping innervation and because measurements of sensory function are usually indirect. For this reason, recovery of motor function is a better criterion, even though it returns somewhat more slowly than sensory function (Medinaceli et al., 1982). However, the interpretation of the data from these evaluations requires an understanding of the functions of peripheral nerve components and the various factors that influence regeneration. Furthermore, an understanding of the normal innervation of skin and muscle and receptors is essential before one can fully appreciate the phenomenon of denervation and long term reinnervation following a nerve injury. Only then can the proper diagnosis and treatment of peripheral nerve injuries be made in the clinical environment.

1.2.2 Approach to the problem

Advances in microsurgery over the last 20 years allow surgeons to realign nerve ends using either the end-to-end anastamosis or fascicular suture repair techniques if the nerve defect or injury does not extend over several millimeters. This type of repair increases the probability of regenerating axons encountering an appropriate distal neural pathway, usually made up of empty endoneurial tubes. If the injury is extensive and if more than ten millimeters of the peripheral nerve is damaged, then the preferred technique is to use a nerve bridge or a nerve graft. Considerable experimental research has been conducted on peripheral nerve repair using the nerve bridge technique. Most of this research has involved the use of single-lumen nerve guides of synthetic or biological origin. In this model system, the lumen of the
tube serves to separate degenerating material that may cause physical obstructions to the regenerating tissues, and forms a controlled experimental chamber where individual peripheral nerve components can be studied under normal or experimentally modified conditions. Lundborg et al. (1981) provoked rats to form subcutaneous mesothelial tubes reinforced with wire coils and then used the tubes to repair severed nerves. The same investigators also used silicone tubes and defined the early sequence of events within the chamber that led to effective regeneration (Lundborg et al., 1982a, 1982b). They described the initial formation of a fibrin-rich extracellular matrix containing blood-derived cells, followed by invasion of endothelial, fibroblastic, and Schwann cells from both ends of the tube, and finally by nerve fibers which form a centrally positioned, vascularized cable enclosed within a connective tissue epineurium and usually separated from the inner wall of the nerve guide by a fluid-filled space. Other investigators have described the organization of constituents within the nerve cable (Uzman and Villegas, 1983), the spatial and temporal progress of the regenerative elements within the regenerative chamber (Williams et al., 1983), the influence of gap length (Lundborg et al., 1982a), effects of mechanical support during nerve transection (Medinaceli and Freed, 1983), and the accumulation of trophic factors within such prostheses (Williams and Varon, 1985; Williams et al., 1987; Danielsen et al., 1988a, 1988b; and Aebischer et al., 1988). Other synthetic nerve guide materials such as polylactate and polyglycolate copolymers (Henry et al., 1985), acrylic copolymers (Aebischer et al., 1990), polyglactin mesh (Molander et al., 1983), MilliporeR filter material (Bassett et al., 1959), GoretexR (Young et al., 1984), and polyethylene terephthalate (Yoshii et al., 1987) have also been used. The growth environment inside the nerve guides has been experimentally modified by
filling the guides with various substances such as laminin (Madison et al., 1985, and 1987), collagen-glycosaminoglycan copolymer matrix (Yannas et al., 1985), collagen (Shine et al., 1985; Satou et al., 1986), collagen and laminin gels (Valentini et al., 1987; Madison et al., 1988; Kljavin and Madison, 1991), dialyzed plasma (Williams et al., 1987), and fibroblast growth factors (Aebischer et al., 1989b, Cordeiro et al., 1989).

The studies using the single-lumen nerve guides with smooth luminal walls show a tapering in the diameter of the regenerating nerve cable bridging the gap between the proximal and distal stumps. One reason for this tapering is because the number of axons regenerating across the injury site are fewer than the axonal fibers making up the proximal stump of the regenerating nerve. Another reason for the thinning of the nerve cable bridging the gap is the lack of mechanical support or a suitable substrate for regenerating axonal growth tips inside the guide. Lundborg et al. (1981) suggest that the central tapering may be due to longitudinal stress lines in the center of the nerve guides, and that the central connective tissue matrix forms the most easily penetrable pathway with good adhesiveness for the regenerating axonal sprouts. Another critical event in regeneration across the gap in single-lumen nerve guides is the formation of a fibrin cable bridge which serves as a scaffold for migrating cells and elongating axons. The growth of this bridge inside the guide is highly distorted owing to the random growth of intra-neural connective tissue elements and to the extra-neural connective tissue.

The present study was undertaken in an attempt to address some of the problems of central tapering, the lack of mechanical support at the middle of the gap, and random orientation of the connective tissue bridge. The multiple-lumen configu-
ration for the nerve cuff proposed in this research increases the surface area available for cellular attachment by approximately 1.7 times as compared to a single-lumen cuff of similar length used as a control. The pattern of holes in the multiple-lumen guide provides a longitudinal orientation for the advancing connective tissue matrix and proliferating Schwann cells, and the regenerating axons follow the Schwann cells in the pre-established pattern. The contribution of the multiple-lumen nerve cuff can be used to directly shape the scaffold of vascular and fibrocytic elements that regenerate from both nerve stumps, as well as to confine the macromolecular extracellular matrix, and possibly to offer three essentials, namely, orientation, substrate, and guidance, for the outgrowing Schwann cell and axonal units.

1.2.3 Significance of the research

The multiple-lumen nerve guide used in the in vivo repair of rat sciatic nerve as described in this study is the first attempt to develop a multiple-lumen nerve cuff to bridge a gap for peripheral nerve repair. This research provides an opportunity to see the effects of a multiple-lumen nerve guide on morphological parameters of regeneration such as axon diameters, total regenerated nerve diameters, total axon cross-sectional areas, spatial arrangement of axons, and the ability of the regenerated axons to form fascicles. The morphological parameters provide a basis for comparison between the experimental multiple-lumen nerve guide and the control single-lumen nerve guide and end-to-end repair method and help to determine if the multiple-lumen cuff either enhances or retards axonal regeneration as compared to the controls. Finally, the observations made from these in vivo studies help in determining the feasibility of using a multiple-lumen nerve guide for peripheral nerve regenera-
tion and provide a basis for making recommendations for further studies in order to better understand the cellular mechanisms underlying the success of peripheral nerve regeneration.
2. LITERATURE REVIEW

2.1 Background

2.1.1 Anatomy of a mammalian peripheral nerve

The primary unit of the mammalian peripheral nerve is the nerve cell, or neuron. Each neuron is made up of dendrites, a nerve cell body (or soma), an axon, and a terminal sensory end organ or motor end plate, depending on the neuron’s function. A peripheral nerve is primarily composed of axons. Axons can be classified as myelinated or unmyelinated, depending on whether they have a coating of myelin. Myelin is made up of a lamellar wrapping of the plasma membrane of Schwann cells. The Schwann cells form the Schwann sheath, which is composed of circumferentially wrapped layers of myelin around the axon. The region of the exposed axon at the junction between Schwann cells is called the node of Ranvier. These nodes are located at discrete intervals along the whole length of the myelinated axon. The connective tissue encompassing the axon and its sheath is called the endoneurium, which is constituted mainly of collagen fibrils, fibroblasts, and macrophages. The perineurium, which is composed of collagen and elastic fibers, surrounds groups of axons and their associated structures, forming nerve bundles or nerve fascicles. The multilayered perineurium serves as a diffusion barrier between axons and extra-axonal elements, and
gives the nerve fascicles most of their tensile strength and elasticity. Areolar con­
nective tissue and collagen fibrils surround the entire nerve. This layer of connective

tissue is called the epineurium (Jenq and Coggeshall, 1986). See Figure 2.1 for a draw­
ing of the typical mammalian peripheral nerve and its components. The peripheral
nerve's blood supply enters via a mesoneurium, similar to the intestinal mesentery.
A well developed system of collaterals between layers and various segments of the
nerve exist within the nerve's blood supply, thereby ensuring a considerable margin
of safety even if the vascular supply is compromised (Swaim, 1987).

Figure 2.1: Schematic representation of the structure of a typical mammalian pe­
ripheral nerve (Swaim, 1987)
2.1.2 Nerve degeneration

Nerve degeneration and regeneration occur simultaneously after a peripheral nerve has been damaged or severed; however for sake of clarity, they will be presented here separately. The most widely accepted nerve degeneration theory is that proposed by Waller, which states that degeneration starts in the part of the nerve that gets severed from the soma, which is the trophic center of the neuron. Regeneration starts from the part of the neuron that is still connected to the neuron's soma. After transection, several factors such as the site of the lesion or severance, the age of the individual, the gap width, the length of the nerve damaged, the alignment of the cut surfaces of the nerve stumps, and the extent of damage and hemorrhage in surrounding tissue influence the growth and development of the regenerating nerve (Seckel et al., 1984; Swaim, 1987).

Hemorrhage and clot formation occur between the cut ends immediately after nerve transection. The accumulation of blood serum and plasma and acid mucopolysaccharides (which have an affinity for water) at about 0.5 to 1 cm on both sides of the transection causes a marked swelling of the nerve stumps within an hour. The swelling slowly subsides after about a week.

The degeneration that occurs in the proximal nerve stump is not as extensive as that in the distal stump. The proximal degeneration is called traumatic degeneration, and it does not reach beyond the second or third node of Ranvier from the injury site. However, damage to axons, myelin, and connective tissue may extend beyond several centimeters on both sides of the injury site when trauma is extensive, like for example, from a high speed missile. The survival of the neurons is proportional to the distance from the cell body at which axonal damage or severance occurs.
Axons and their myelin start degenerating when the distal stump undergoes Wallerian degeneration. For about 2 weeks, the transected axon sections at the proximal end of the distal stump enlarge and get isolated from the rest of the distal stump. However, the remaining axonal sections in the distal stump degenerate more rapidly, and axonal and myelin sheath degeneration becomes evident along the distal stump by the end of 48 hours. The laminated or layered organization of the myelin sheath is lost, and the degenerating myelin becomes homogeneous and forms ovoids and ellipsoids, surrounding degenerating axonal segments. The axoplasm increases in optical density and coalesces, forming clumps, while the neurofibrils degenerate and disappear (Swaim, 1987). Macrophages from intra-neural and extra-neural sources move into the degenerating area and remove the products of degeneration, leaving intact only the connective tissue framework of the distal nerve stump. See part A in Figure 2.2 for details of Wallerian degeneration. These macrophages start appearing at the injury site about 7 days after injury, and this invasion reaches a peak at about the third week after injury. Schwann cells also have been known to play a key role in phagocytizing axonal and myelin breakdown products. This debris removal process continues for about 21 to 56 days after injury. During this time, the distal nerve stump gets less swollen, and the phagocytic activity slowly subsides. Endoneurial sheaths shrink and disappear, and the newly regenerated neurilemmal sheaths realign in an orderly fashion. The distal nerve stump gets progressively contracted and replaced by connective tissue if the regenerating axons from the proximal stump do not invade it.
2.1.3 Nerve regeneration

The regeneration process involves the peripheral growth of new axons from the proximal stump of the nerve, across the injury or severance site. The neuron's soma or cell body expends considerable energy during this process. The process has its origins within the soma of the severed axon and is similar in both sensory and motor nerves. Chromatolysis (i.e., disintegration of the Nissl bodies) occurs in the nerve cell body, causing it to progressively enlarge for about 10 to 12 days. The soma remains swollen during the entire active regeneration period and returns to normal size as maturation progresses. There is an increase in both RNA and DNA activity within the neuron during this period. The metabolic activity also increases within the cell due to increased enzymatic activity and incorporation of amino acids. This heightened activity may result in a neuron replacing 50 to 100 times the protein.
and organic material that is usually contained in the normal soma. The increased metabolic activity in the neuronal soma is also aided by the alterations that occur in the glial cells surrounding the neuron. The swelling of the neuronal soma peaks early in the regeneration process and again when myoneural junctions are formed. The changes are proportional to the proximity of the damage and are more pronounced when the damage or severance site is closer to the soma. However, the cell body may die if the injury is very close to it, or even if it survives, the amount of new axon that must be produced may exceed the metabolic capacity of the cell and will result in axons failing to regenerate.

New protoplasm that is synthesized by the neuronal soma is transported by axoplasmic flow from the cell body down the axon. This transport has a slow and a fast component. The slow component involves microperistalsis within the nerve membrane and the fast component involves the microtubules. Transport by microperistalsis occurs at the rate of 1 mm/day and by microtubules at about 10 cm/day, which is 100 times faster. A part of the slowly transported proteins, during the passage down the axon, is utilized to replace enzymes that have been catabolized in the membrane. Nevertheless, a major fraction reaches the terminal regions of the regenerating axon (Swaim, 1987). The microtubules also play a significant part in the fast transportation of new axoplasm to regions of increased nutrient requirements and metabolic activity, such as synaptic sites. See part B in Figure 2.2 for details of nerve regeneration.

The changes that occur in and between the proximal and distal nerve stumps after nerve transection strongly influence nerve regeneration. The rate of proliferation and organization of the epineural and endoneural connective tissue, Schwann cells,
and blood vessels (mainly capillaries) strongly influence the regeneration of axons across the injury site (Williams et al., 1983; Lundborg et al., 1981). Proliferation of fibroblasts (of epineural origin), connective tissue, and capillaries begins within 1 to 3 days after nerve severance or damage from both the stumps. These regenerating tissues infiltrate the injury site and migrate toward each other and establish a tissue bridge and capillary bed between the proximal and distal nerve stumps, thereby creating an environment for axonal regeneration. From 4 to 20 days after nerve damage, the regenerating axons start sprouting from the proximal stump, in conjunction with the heightened metabolic rate of the neuron's soma. The location of the start of sprouting or budding of the regenerating axons depends on the type of injury. Budding begins at 1 to 3 cm proximal to the severance site in cases of wide-spread traumatic injuries and begins a few millimeters retrograde to the last node of Ranvier in cases where the injury is sharply localized (Swaim, 1987).

Schwann cells also play a key role in axonal regeneration. They proliferate at much higher rates and greatly outnumber the connective tissue cells of endoneural and perineural origin during the early stages of regeneration. As they proliferate, the Schwann cells form longitudinally oriented bands of Büngner, which are contiguous with the pre-existing Schwann tubes in the nerve stumps. Eventually the Schwann cells of each stump join as they grow toward each other. Since the Schwann cells of the proximal stump slightly precede those advancing from the distal stump, the proximal Schwann cells serve as a guidance mechanism for the regenerating axons (Aguayo et al., 1979; Lundborg et al., 1982a; Seckel et al., 1984).

The regenerating axons branch several times as they cross the repair site and these sprouts have a natural affinity, called homotropism, for Schwann cells. This
attraction causes the regenerating axons to follow a Schwann cell Büngner band through an empty endoneural tube in the distal stump. Here, the Schwann cells envelope the regenerated axon by multiple concentric wrappings, thereby remyelinating the axon. Remyelination also occurs at the injury site, and as the newly myelinated axons infiltrate the persisting endoneural tubules in the distal stump, they push the Schwann cells of the tubule to the side. Although several axonal branches may infiltrate a tubule and develop peripherally, only one branch will become myelinated and matures fully once successful contact is established with a peripheral end plate. Scanning-electron microscopic studies by Gershenbaum and Roisen (1978) have confirmed that Schwann cells form new endoneural tubes during regeneration and that the regenerating axonal sprouts make use of either the new or old pre-existing endoneural tubes to regenerate in the distal stump. However, most myelinated and unmyelinated regenerated axons seem to favor the newly formed endoneural tubes while progressing peripherally.

The rate of axonal regeneration at the periphery of the repair site is about 0.25 mm/day. Once clear of the repair zone, the regeneration rate increases to 1 to 4 mm/day, or approximately 2.5 cm/month. In spite of a 3 to 4 mm/day rate of regeneration for axonal tips, the functional recovery rate is only about 1 to 2 mm/day. The axonal regeneration rate varies during the course of regeneration in a nerve, with lag periods at the start and end of the regeneration process. Rates are higher in the proximal areas and lower in the more distal areas. Also, the condition of the motor end plate and the health of the muscle fibers being re-innervated determine the success of axonal regeneration and degree of functional recovery established (Swaim, 1987). Hence, physical therapy and care of muscle and skin in the denervated region are
vital for peripheral nerve regeneration.

2.2 Repair techniques and review of previous work

2.2.1 Requirements for a peripheral nerve bridge

The method of nerve repair chosen depends upon type of injury and the gap length. End-to-end anastomosis is the preferred method for gap lengths less than 10 mm. Indications for nerve grafting vary from gaps of 10 mm to over 50 mm before a graft can replace an end-to-end repair under tension. When a graft is indicated, autografts are preferred at the present time. The autograft satisfies three major requirements for an ideal nerve graft:

- it serves as a passive conduit for axonal regeneration,
- it is a natural substitute which is immunologically acceptable, and
- it is easily vascularized by the recipient bed.

The biggest drawback of the autograft is the requirement of a donor nerve for grafting and the added risks of surgery at another site. Homografts (a graft of tissue between individuals of the same species, but of disparate genotype, also called allo-graft), heterografts (a graft of tissue transplanted between animals of different species, also called xenograft), and artificial nerve graft substitutes which have been studied as an alternative to autografts have been found to be immunologically unacceptable (Rosen et al., 1989). These two major problems can be solved by developing an artificial nerve guide or channel for peripheral nerve repair. For use as ideal nerve grafts,
the bridging devices, which represent a wide range of biological and non-biological materials, must fulfill the following eight requirements:

- they must serve as a conduit/scaffold for axonal regeneration,
- they must be immunologically acceptable,
- they must be vascularized by the host so that regenerating axons have a blood supply,
- they should eliminate or minimize tissue reaction,
- they should reduce disorderly proliferation of fibroblasts and connective tissue surrounding the injured nerve,
- they should minimize scar formation,
- they should contain a neurotrophic substrate hospitable to growing nerve fibers, and
- they must be readily available.

2.2.2 Various repair techniques

There are two basic approaches to peripheral nerve repair, the anatomical approach and the cellular approach. The aim of the anatomical approach is to re-establish continuity of the various layers of tissue. The conventional suture and nerve bridging methods reconnect the epineural or perineural layers of connective tissue to permit healing of the nerve. This kind of repair does not separate the fibrous healing process of the surrounding connective tissue from the healing processes of the nerve
tissue. The following methods are based on the anatomical approach. The models based on the cellular approach are explained later in this section.

Nerve defects can be healed by using tubular implants of autogenic (tissue arising, transferred, or transplanted within an individual) or synthetic origin (Lundborg and Hansson, 1980). Chiu et al. (1982) used a segment of femoral vein to bridge a gap of one cm in the rat sciatic nerve. An orderly arrangement of regenerated fibers within the lumen of the vein graft reached the distal stump within 2 months after surgery, and some restoration of nerve conduction and muscle reinnervation was achieved. By 16 weeks, in vivo nerve conduction studies showed a conduction velocity of 16.3 meters/second in the vein grafted animals as compared to 64.5 meters/second in normal control animals.

In 1983, Molander et al. used a polyglactin suture mesh (pore size of 0.4 x 0.4 mm and a thread diameter of 140 ± 20 μm) to bridge a gap of 10 mm in adult rabbit tibial nerve and compared it with conventional nerve grafting. There were no statistically significant differences between the two groups. The regenerating axons were confined within the newly formed epineural sheath inside the polyglactin tubes. They concluded that polyglactin tubes bridging a fresh nerve defect of moderate size give regeneration as good as conventional nerve grafting.

The nerves to the medial gastrocnemius muscle and the lateral gastrocnemius and soleus muscles in cats were severed and crossed using a GoretexR tube (internodal distance = 30 μm) by Young et al. (1984). Four to fifteen months post-operatively, they showed that the GoretexR sleeve was effective in directing nerve fiber growth to the respective muscles that they innervated. Electrophysiological studies of muscle force and dorsal root volleys showed a complete absence of unintended reinnervation.
and a regeneration that was more substantial for motor than sensory axons. Histology showed no evidence of regenerating nerve tissue invading the Gore-tex® wall.

In 1985, Madison et al. used bioresorbable synthetic nerve guides made of poly-D,L-lactates to bridge gaps of 5 mm in adult mice sciatic nerves. These guides were filled with a gel containing 80% laminin and other extra-cellular matrix components such as type IV collagen, and heparan sulfate proteoglycan in the molar ratio 1:1:0.1. Laminin (an abundant component of basement membranes) had been previously shown by them in in vitro studies to act as a preferred substrate as well as a stimulatory agent for peripheral and central axonal regeneration. Two weeks post-operatively, the investigators observed a significant increase in the speed of axonal regeneration within the laminin-coated guides.

In 1987, Luis Cuadros and C. E. Granatir used a synthetic microporous expanded polytetrafluoroethylene tube (pore size = 30 μm, 0.1 mm wall thickness) to bridge a 10 mm gap in the sciatic nerve in rats and compared these animals with a control group which was repaired using a autogenous nerve graft. Four months after repair, nerve regeneration in the polytetrafluoroethylene (PTFE) tubes was equivalent to that seen in the autogenous nerve grafts. Nerve fibers were organized into coherent bundles inside the PTFE grafts. No fibers were seen within the graft wall itself and the inner surface of the PTFE was separated from the nerve fibers by a thin, fibrous layer. The most significant histological finding was abundant neo-vascularization and capillary ingrowth through the microporous PTFE wall which allowed nutrients to reach the regenerated cable directly through the wall of the tube. Nerve conduction velocity at 4 months in the PTFE tubes was 21.7 meters/second as compared to 50.6 meters/second in normal control nerves.
Yannas et al. (1985, and 1989) used a silicone tube filled with a highly porous, biodegradable collagen-glycosaminoglycan (CG) copolymer matrix to study regeneration of axons across a 10 mm gap in the rat sciatic nerve. Degradation rate, average orientation of pore channel axes, and average pore diameter of the CG matrix were varied independently during processing of the matrix, and their effects on nerve regeneration were studied separately from each other. They achieved the best regeneration when the matrix degraded rapidly, as well as when the pore channel axes were oriented axially, rather than radially, along the graft, and when the average pore diameter of the matrix was 5 μm. They did not provide an explanation for the axon-biomaterial interactions and the substrate preference shown by the regenerating axons.

Satou et al. (1986) have reported that silicone tubes which were filled with a collagen gel (Cell Matrix II) accelerated the growth of sprouting axons in the initial stages, inhibited proliferation of fibroblasts, and kept the direction of proliferating Schwann cells under control. According to them, these all are crucial factors in axonal regeneration.

Williams et al. (1987) demonstrated that using a silicone chamber pre-filled with dialyzed plasma to bridge a 15-mm gap in the adult rat sciatic nerve resulted in a 3.5 fold increase in the restitution of function detected at eight weeks post-implantation as compared to the results using chambers pre-filled with phosphate-buffered saline (PBS). The diameter of the regenerate was 1.6 times greater in the experimental groups compared to the control PBS groups. Also, a muscle action potential was detectable in 75% of the experimental animals compared to 20% of the PBS controls.

Müller et al. (1987) evaluated the effects of exogenous biochemical agents such
as laminin, testosterone, ganglioside GM1, and catalase applied by multiple injections into silicone nerve regeneration chambers. At 16 days post-implantation, they observed a 2 fold increase in blood vessels, 3 fold increase in Schwann cells, and a 10 fold increase in axons at the center of the chambers for the experimental animals compared to saline injected controls.

Valentini et al. (1987) used collagen (type I or type III) or laminin gel filled semipermeable (50,000 Da molecular weight cutoff) polyvinylchloride acrylic copolymer guidance tubes (wall thickness = 0.15 mm) to repair a 4 mm sciatic nerve gap in mice. At 12 weeks post-implantation, they observed fewer myelinated axons in the gel filled tubes than in the control saline filled tubes. They concluded that the gel substrate, even if it contained neurite-promoting factors, impairs the regeneration process by physically impeding the diffusion of critical molecules, migration of cells, and the elongation of axons. They suggested that the positive effects (such as concentrating high-molecular weight endogenous growth factors inside the regenerating chamber) induced by the semipermeable guides were negated by the growth substrates in gel-form added to the lumen of the guides.

Le Beau et al. (1988a) conducted both in vitro and in vivo experiments to establish the growth promoting effects of the extracellular fluid surrounding rat sciatic nerve during regeneration using a silicone tube. The extracellular fluid enhanced Schwann cell adhesion, migration, and proliferation during peripheral nerve regeneration and remyelination. The same researchers (Le Beau et al., 1988b) also used light and electron microscopy to investigate the ultra-structure and to conduct morphometric analysis on long-term peripheral nerve regeneration of rat sciatic nerve through a silicone tube. They reported that even after 77 weeks, complete matura-
tion of many regenerated axons was never achieved. They noticed axonal distortion by neurofilaments, axonal degeneration and secondary demyelination after 8 weeks following nerve transection. These changes progressed in severity with time as more axons advanced through the distal stump to reach peripheral targets. Quantitative evaluations showed that mean axon diameters were significantly lower than normal, mean axon diameters did not increase much after 6 weeks, myelin sheath thickness increased with time, and the number of regenerated fibers decreased in some cases over extended periods.

Kljavin and Madison (1991) used a non-permeable Tygon R tube filled with either a collagen or laminin matrix to bridge a 4 mm nerve gap in mice. They found that early axonal ingrowth was greater in tubes filled with a laminin containing gel as compared to collagen filled tubes. Axons also grew longer distances in the laminin filled tubes. However, cellular ingrowth was more dense and compact in the collagen matrix. More cells grew into the collagen matrix, but for shorter distances compared to growth in the laminin matrix. They suggested that their observations concerning early axonal ingrowth were due to the ability of cells to migrate more rapidly through the laminin as compared to the collagen matrix. These rapidly advancing cells then provided a suitable guidance mechanism for axonal elongation.

Rosen et al. (1989) compared the regeneration of rat peroneal nerve across a 5 mm gap repaired with a sutured autograft versus an artificial nerve graft. The artificial nerve graft was made of biodegradable polyglycolic acid and the lumen of the graft was filled with a synthetic growth medium composed of hypoallergenic collagen (mainly collagen type I and some collagen type III). Qualitative histological evaluations showed the organization of repair sites in the artificial nerve graft was worse
than that observed in the sutured autografts for long term animals. Quantitative histological evaluations conducted to measure mean fiber diameters at sections 5 mm distal to the repair site did not show a significant difference between the two groups. Quantitative electrophysiology was performed to measure the Integrated Monophasic Compound Action Potential (IMCAP) in both groups. The IMCAP percentages provided a measure of regeneration which served as a comparison, but did not provide an absolute percentage of the number of axons that had functionally reconnected across the repair site. Statistical tests showed a significant improvement in the IMCAP percentages for the sutured autograft repair as compared to the artificial graft repair, denoting that the regaining of physiologic function in nerves repaired with artificial nerve grafts was inferior to that in nerves repaired with the sutured autograft.

An alternative to this anatomical approach to nerve repair is a cellular approach. The aim of this method is to separate the specific cellular components of the peripheral nerve that contribute to fibrous healing and nerve regeneration. The perineurium divides the peripheral nerve into two distinct cellular environments, the intrafascicular and extrafascicular environments. The intrafascicular environment comprises the cellular components like Schwann cells, axons, and endoneural fibroblasts that constitute the nerve's regenerating unit. The extrafascicular environment encompasses the cellular components like epineural and extraneural fibroblasts which are crucial to the fibrous reaction to injury.

A cellular approach uses a tube or guide around the fascicles. The tube acts as an artificial perineurium to separate the intrafascicular nerve tissue from the extrafascicular connective tissue. This provides a way to separate fibrous healing from axonal regeneration until the natural perineurium reestablishes its continuity. Some of the
problems like overriding, gapping, buckling, and straddling of the fascicles associated with the conventional end-to-end anastamosis repair technique (which uses sutures to appose the outermost epineurium) can be minimized or eliminated by the cellular repair technique. Rosen et al. (1979, 1983) and Marshall et al. (1989) demonstrated the fascicular sutureless and suture repair techniques in the peripheral nerve repair of rats. They were not able to conclusively prove this technique to be superior to conventional epifascicular suture repair.

2.2.3 Summary of past research

For over a hundred years researchers have attempted to find an ideal medium for axonal regeneration and passive devices for bridging gaps resulting from resected nerves. In 1944, Paul Weiss (1944) reviewed previous studies of artificial nerve grafts. Since then, researchers have used various artificial nerve guide materials like polyethylene, polyglycolic acid, polyglactin, poly-lactates with plasticizers, Goretex\textsuperscript{R} (expanded PTFE) and silicone rubber. Autografts, such as autogenous veins, have also been used as passive conduits for nerve regeneration. They were acceptable, but were not an ideal artificial nerve graft (Chiu et al., 1982). With varying degrees of success, researchers also have used pseudosynovial or preformed mesothelial tubes as nerve grafts (Lundborg and Hansson, 1980; Lundborg et al., 1981; Lundborg et al., 1982b). Other authors have experimented with tubes of various synthetic resorbable materials (like collagen) and non-resorbable materials for peripheral nerve repair (Cuadros and Granatir, 1987; Bassett et al., 1959; Henry et al., 1985; Rosen et al., 1989, Uzman and Villegas, 1983). They concluded that laminin, collagen, and other neuronotrophic factors, such as fibroblast growth factors, enhanced peripheral
nerve regeneration by increasing numbers of regenerated axons in the repaired nerve. Gibson and Daniloff (1989) compared nerve regeneration through silicone tubes and nerve allografts. Regeneration was assessed at 10, 24 and 90 days post-implantation. At 90 days, they noted that the nerve graft group had markedly superior conduction velocity times as compared to the silicone implant groups. They suggested that as the nerves regenerated and enlarged, the encircling silicone tube led to compression of the nerve trunk and to an overall decrease in return of function.

It is now established that the regenerating nerve fibers can both influence, and be influenced by, the cellular and extracellular components of their immediate environment. The effect of the environment on the rate and extent of peripheral nerve regeneration is now considered to be critical (Yannas et al., 1989). It has also been determined that Schwann cells play a key role in the regeneration of the proximal stump towards the distal stump across a gap (Shine et al., 1985). Work done by Aguayo et al. (1979) and Bunge and Bunge (1978), using Schwann cells grown in tissue culture and transplanted into peripheral nerves, has shown that the Schwann cells do ensheathe and remyelinate axons.

Lundborg et al. (1982a) demonstrated the importance of the presence of the distal nerve stump and the influence of the gap length on peripheral nerve regeneration in rat sciatic nerve. They used a silicone chamber to bridge gap lengths of 6 mm, 10 mm, and 15 mm in the rat sciatic nerve. The influence of the distal stump in peripheral nerve repair was studied by implanting some silicone chambers in which both proximal and distal stumps were inserted into the ends of the chamber, and other chambers in which only the proximal stump was inserted and the distal end was left open. Axonal growth occurred in the 6 mm gap chambers with and without
distal stumps. In the 10 mm gap chambers, only the chambers with both the stumps displayed fully regenerated axons, whereas the chambers without the distal stump showed no or partially regenerated axons. No regeneration was seen in the 15 mm gap chambers at all. The researches concluded that the distal stump influences axonal regeneration over a limited distance or volume. They supposed that the influence could be through humoral agents released from the distal stump. This observation was later confirmed by Politis et al. (1982), Williams et al. (1984), Seckel et al. (1984), Yannas et al. (1985), and Madison et al. (1985) who used similar silicone chamber models.

Williams et al. (1984) tested the ability of skin, tendon, and sciatic nerve (an isolated 2 mm piece) to support axonal regeneration in a silicone chamber. They reported that the isolated sciatic nerve insert stimulated fibrin matrix formation, cell immigration, and axonal regeneration, whereas the skin and tendon inserts did not encourage cell migration and axonal elongation inside the silicone chamber. They concluded that fibrin matrix formation and a cellular bridge are vital precursors for axonal regeneration. Successful regeneration required humoral and/or cellular contributions which were available from peripheral nervous tissue and not from the other tested tissue.

Glasby et al. (1986) and Keynes et al. (1984) have demonstrated the influence of basement membranes on peripheral nerve regeneration. Glasby et al. (1986) used an autogenous muscle graft to bridge a gap in rat sciatic nerve. Seven weeks after implantation, they observed well developed myelinated nerve fibers within the graft and in the distal stump. Normal axon numbers were achieved in the grafts by 12 weeks. However, the regenerating nerve in the muscle grafts took 6 months to
a year to recover normal axon diameter and myelination. Recovery was delayed in muscle grafts whose basement membrane tubes were perpendicular to the nerve fibers in the proximal stump. They concluded that regeneration depends on availability and orientation of empty basement membrane tubes. Keynes et al. (1984) used muscle bridges to repair cut sciatic and saphenous nerves in adult female mice. By 4 days they saw axonal growth parallel to the length of the muscle fibers, and this coincided with the onset of degeneration of the sarcoplasm. At 10 days, over 90% of the regenerated axons were located inside the sheaths of muscle fiber basement membranes.

Finally, attempts have been made to quantitatively evaluate the effects of different repair techniques on peripheral nerve regeneration. Jenq and Coggeshall (1985, 1986, and 1987) have defined numerical parameters of axonal regeneration in peripheral nerves. They have used morphometric parameters such as numbers of unmyelinated and myelinated axons, cross-sectional areas of the regenerated nerve, and density of blood vessels to evaluate the results of their repair methods. Other quantitative parameters such as endoneural and blood vessel cross-sectional areas (Danielsen et al., 1988a), myelin sheath thickness (Le Beau et al., 1988a), axon density, ratio of total axonal area to total area of the regenerated nerve (Lundborg et al., 1982b), and percentage area occupied by neural components such as epineurium and perineurium (Uzman and Villegas, 1983; Müller et al., 1987) have also been utilized to quantify regeneration. To evaluate the extent of remyelination, the myelin thickness has been usually expressed as the ratio of fiber diameter to axon core diameter (also known as the g-ratio) by Fields and Ellisman (1986b), Danielsen et al. (1988b), Henry et al. (1985), Le Beau et al. (1988b), and Lundborg et al. (1982b). The distribution of the
different fiber types and fibers at different stages of regeneration and remyelination have been represented as a fiber diameter histogram (FDH) or as axon distributions by several researchers (Lundborg et al., 1982a; Marshall et al., 1989; and Rosen et al., 1983, 1989). Quantitative electrophysiological results have been expressed as conduction velocity measurements, refractory periods, and time constant of excitation by Chiu et al. (1982, 1988), Cuadros and Granatir (1987), Fields and Ellisman (1986a), and Yannas et al. (1989). Functional recovery in the distal stump has also been evaluated by the ratio of compound action potential (CAP) areas in the distal stump to the CAP areas in the proximal stump by Ashur et al. (1987), Marshall et al. (1989), Rosen et al. (1979) and Sabelman et al. (1989).

2.3 Factors influencing peripheral nerve regeneration

2.3.1 Influence of neuronotrophic factors

Until about 1945, neurobiologists thought that end-to-end anastomosis of the proximal and distal stumps was essential for successful peripheral nerve regeneration. This was based on studies done by Waller in 1850 on the degenerative changes that occur in the distal nerve stump following transection of a peripheral nerve. Waller stressed the importance of the nerve cell body as a trophic or nutritive center that supported the distal nerve's existence, function, and regenerative capabilities. It was the discovery of nerve growth factor (NGF) by Levi-Montalcini and Hamburger in 1951 (Levi-Montalcini, 1987), and its subsequent purification by Stanly Cohen (Cohen, 1960), that revolutionized the study of nerve regeneration and changed the long held Wallerian theory of the nerve cell body as being the only trophic center of the neuron.
In the past thirty years, there has been a large increase in the research done on the various factors external to the nerve cell that influence its development, growth, survival, and regenerative abilities. These factors, which are called neuronotrophic factors (NTF), are macromolecular proteins that are essential for the growth and survival of various classes of nerve cells in tissue culture. These factors can be either humoral (i.e., pertaining in this case to fluid involved with peripheral nerve regeneration) or substrate-bound. In the adult mammalian model, the humoral factors are released by the target organ or structure to be innervated. These are taken up by the regenerating axons and are transported in a retrograde fashion to the soma of the neuron where they exert their trophic effects. Substrate-bound neuronotrophic factors, which are also secreted by the distal targets of innervation, coat the surface on which the proximal regeneration axons are growing. They lack a survival-promoting function, and they are vital for the growth or extension of neurites. Additionally, factors which are closely associated with the substrate on which axons regenerate, and possibly factors released from blood vessels, are also thought of as being crucial to the regeneration process.

The neuronotrophic factors (NTF) play a key role in the growth of peripheral axons. Also several other natural components of the peripheral nerve presumably play an important role in not only peripheral nerve regeneration, but also central nervous system cholinergic neuronal regeneration (Varon et al., 1991). Their purification and incorporation in the growth medium will be a crucial step toward the manufacture of an ideal growth medium.
2.3.2 Influence of regenerating environment

The interaction of regenerating neural processes with the surface or substratum on which they grow is another important factor to be considered in developing a nerve guide or channel for peripheral nerve repair. In reporting the work done on regenerating nerves in pseudosynovial tubes, Lundborg and Hansson (1980) showed that the serum harvested from inside the mesothelial chamber has a strong neurotrophic influence on the growing neurites in tissue culture. Williams et al. (1983) showed that if the regenerating proximal stump is placed in an appropriate environment, it can form a well-organized and oriented nerve trunk. Danielsen et al. (1988b) showed that a silicone nerve regeneration chamber that is partitioned into two compartments by a strip of nitrocellulose paper treated with a basic fibroblast growth factor (b-FGF) solution stimulates peripheral nerve regeneration by markedly increasing migration of perineural-like cells, vasculature and Schwann cells. Aebischer et al. (1989b) investigated the ability of ethylene-vinyl acetate copolymer nerve guidance channels which released sustained controlled amounts of basic fibroblast growth factor (b-FGF) and/or alpha-1 glycoprotein (α1-GP) to support rat peripheral nerve regeneration over a 15 mm gap. Four weeks after implantation, the synthetic tubes releasing b-FGF or b-FGF and α1-GP exhibited both myelinated and unmyelinated axons which bridged the gap between the nerve stumps. They had two main conclusions. Controlled release of b-FGF and α1-GP enhanced peripheral nerve regeneration. Synthetic nerve guides that released macromolecules in a controlled manner served as an useful tool in the study of peripheral nerve repair. The stimulatory effects on regenerating axons by acidic fibroblast growth factor (a-FGF) were studied by Cordeiro et al. (1989). When added to a collagen filled nerve guide, purified
a-FGF not only enhanced axonal branching within the guide but also increased the number of myelinated axons, primary sensory axons, and motor neuronal axons that regenerated across a 5 mm gap. Acidic FGF is the first highly purified growth factor since the discovery and use of nerve growth factor that has been shown to promote peripheral nerve regeneration in vivo. Several other researchers have evaluated the effects of empty and saline filled silicone chambers on the fibrin matrix formation (Williams and Varon, 1985), laminin (Madison et al., 1985, 1987, 1988; Yoshii et al., 1987), and biodegradable collagen-glycosaminoglycan (CG) co-polymer matrix (Yannas et al., 1989) on regenerating axons.

2.3.3 Influence of the physical characteristics of the nerve bridges

Until very recently, artificial channels or guides have only been used as inert conduits to provide guidance to regenerating axons, to prevent invasion of scar tissue, and to maintain an environment conducive to axonal growth. Not much emphasis has been placed on the relationship between the physio-chemical properties of the channel or guide and the results of regeneration. These interactions have to be studied experimentally and should be explained in more detail. Experiments still show that the morphological and functional results of peripheral nerve regeneration compare poorly with the normal situation. It is essential to identify the physical characteristics of nerve channels or guides which optimize peripheral nerve regeneration because this may lead to a better understanding of the healing process, thereby leading to an improved design of these channels or guides for use in the clinical repair of severed or injured human peripheral nerves.

The three most important characteristics of nerve channels-guides that influence
peripheral nerve regeneration are:

1. permeability characteristics,
2. electrical characteristics,
3. and microgeometry.

The channels or guides facilitate the repair of the transected nerve in experimental animals and have shown promise for clinical applications in humans. However, the optimal material for these guides has not been determined. Some of the common biocompatible polymers which have been used to make these channels or guides are silicone rubber (most popular), polyvinyl chloride, polytetrafluoroethylene, polyethylene, acrylic copolymers and bioresorbable polyesters (Aebischer, 1988). The focus has been almost exclusively on the biological events controlling the nerve regeneration process. Not much attention has been directed to the effects that the channel's or guide's physical properties have on the axonal regeneration process. It is also necessary to view the channel or guide as a material which influences the cellular and metabolic aspects of the regenerating process and thereby plays a direct role in the regeneration.

The permeability characteristics of the guidance channel, or guide, directly influence the regeneration by mediating solute exchange between the intra-channel and extra-channel environments. Experiments conducted by Aebischer et al. (1989a), using permselective (a polymeric material which, depending on the molecular weights of the polymeric chains, allows only certain sizes of macromolecules to permeate through it) channels demonstrated that the transport properties of the wall of the
channel can help create an optimal regenerating environment in addition to providing other favorable characteristics such as minimizing the escape of growth factors released at the injury site and preventing the release of nerve antigenic factors. The synthetic permselective materials with a wide range of molecular weight cut-offs give researchers the advantage of studying both intra-channel and extra-channel factors influencing axonal regeneration.

The influence of the channel’s or guide’s electrical properties has been the subject of intense investigation lately (Fine et al., 1991). Ever since in vitro neurite outgrowth has been shown to be stimulated and directed by electrical activity, nerve channels and guides possessing electrical properties have been used to enhance nerve regeneration in vivo. Electrets (dielectric materials in which the electrical charge is stored predominantly as trapped surface charge) and piezo-electric materials (materials which need some form of mechanical deformation to generate a transient electric field) are some of the materials that are being studied currently for use as nerve guides or channels.

The luminal surface morphology and microgeometry of the nerve guide or channel also plays a crucial role in influencing the early arrangement of the fibrin matrix and inducing different cellular responses. In smooth walled synthetic nerve guides, the regenerating bridge usually is centrally located inside the guide and is surrounded by an acellular gel. The nerve cable does not touch the channel’s or guide’s inner luminal surface. Aebischer et al. (1990) have shown that a synthetic guide with a relatively rough trabecular network lining the luminal surface resulted in relatively poorly regenerated nerve fascicles without an epineurium. The growing nerve fibers were suspended in a loose connective tissue which entirely filled the lumen. This
should not be surprising because well characterized rough synthetic surfaces have been shown to induce a stronger tissue reaction than smooth surfaces in both the subcutaneous and intra-muscular locations (Aebischer, 1988). Conversely, the guides or channels with smooth walled lumens contained discrete, free-floating nerve cables with numerous myelinated and unmyelinated axons which were surrounded by a thin, continuous epineural-like layer. When both smooth and rough surfaces lined the luminal wall alternately, the general morphological patterns of the regenerated nerve cable in individual smooth or rough segments were similar to those observed in only smooth or only rough guides respectively. These observations suggest that the morphological arrangement of the regenerated nerve tissue is heavily influenced by the surface microgeometry of the guides or channels. For successful peripheral nerve regeneration, it is necessary to fully understand the influence of surface geometry of the guide or channel on the early events of regeneration and the process of initial cable formation.
3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Silicone rubber

Silicone rubber (polydimethylsiloxane) is one of the most popular biocompatible polymers for implant applications. In general, medical grade silicone rubbers can be subdivided into the heat-vulcanizing variety and the room-temperature vulcanizing variety (RTV). A finely divided silica filler (particle size of about 120 to 300 Angstroms) is used to improve strength in the heat-vulcanizing variety of silicone rubber, and usually, the more the filler used, the harder the rubber is. The RTV rubbers can be further divided into the two component RTV and one component RTV (Braley, 1970). Diatomaceous earth particles ranging in size from 1 to 30 \( \mu m \) are used as filler in the two component RTV rubber. Vulcanization is achieved by adding a catalyst (usually stannous octoate) to the base, which is a fluid silicone polymer, mixed with filler and a cross-linking agent. These cross-linked polymers form the silicone rubber with filler dispersed in the matrix. Vulcanization is initiated in the one component system by absorption of water vapor from the air.

The silicone rubber used in fabricating the 7-lumen tubing was a heat-vulcanizing variety called Q7-4750, manufactured by Dow Corning Corporation (Midland, Michi-
gan). The thermal-setting Q7-4750 medical grade Silastic\textsuperscript{R} elastomer is a two-part, enhanced tear resistant (ETR) silicone elastomer that consists of dimethyl and methylvinyl siloxane copolymers and reinforcing silica. The specific advantages of this elastomer include:

- non-tacky surface,
- no peroxide residues,
- optional post-cure,
- blendable to required durometer,
- no volatile by-products,
- lower cure temperatures, and
- qualified to U.S.P biological standards.

The elastomer is strained through a 200 mesh screen to ensure freedom from particulate contamination, and vulcanization is achieved by curing for 10 minutes at 240\degree F. The catalyst used is platinum in this case. The vulcanized polymer is then post-cured in an oven for 1 hour at 350\degree F.

In the present study, silicone rubber was chosen as the artificial nerve graft material because it is immunologically acceptable with minimal reaction in most cases. The cuff functions as a conduit for the regenerating nerve, provides longitudinal orientation for migrating cells and elongating axons, and insulates the growth process from the extraneural environment.
Most studies on peripheral nerve regeneration have used nerve guides, or channels, which have little or no permeability to watery solutes. The most common material of choice has been silicone elastomer. Silicone rubber, because of its non-permeability, isolates the regenerative environment from the surrounding tissues so that only cells or fluids within the chamber influence regeneration. The wall of the silicone rubber tubing barricades the line of repair against invasion by extraneurial cells. This way the effects of various external factors that influence regeneration are eliminated. Also, the luminal walls present an adequate interface for guidance of the regenerating axons crossing the gap until continuity with the distal nerve is re-established.

Since the main objective of this research is to test the feasibility of using a multiple-lumen nerve cuff for peripheral nerve regeneration, all other factors, such as permeability of material, surface morphology and electrical properties which influence regeneration, are eliminated by using silicone rubber as the cuff material. By choosing silicone rubber and by incorporating a multiple-lumen design, it is possible to study the effects of just cuff geometry on axonal regeneration.

3.1.2 Silver stain methods for neural tissue

The basis of the silver stains is the argyrophilia of the neurofilament proteins of normal axons. Although silver stains are regarded as histochemically specific for neurofilaments, other objects such as nuclei and the cytoplasm of many endocrine cells are also stained. Even though the details of the chemical reactions that occur during silver staining are known, not much is known about the reason for the specificity of the methods (Kiernan, 1990).
All silver stain neurohistological techniques have three common features:

1. treating the fixed tissue with a solution containing silver ions, of concentration between $10^{-5}$ M to 1.0 M

2. treating the specimen subsequently with a reducing agent capable of causing the reaction

\[ Ag^+ + e^- \rightarrow Ag \downarrow \]

3. depositing of a dark, opaque, material consisting mainly or entirely of metallic silver in the argyrophilic axons

In the first step of the process, silver is taken up in two ways. The larger quantity is bound chemically by protein throughout the tissue. This chemically bound silver is not axon-specific. A much smaller amount of silver is reduced at sites in the axons and precipitated as tiny nuclei of the metal. In the second stage of the method, the sections are transferred to a solution similar to an alkaline photographic developer, such as sodium sulphite and hydroquinone. The sulphite removes the chemically bound silver, introducing $[Ag(SO_3)_2]^{3-}$ ions into the solution. Hydroquinone reduces this complex-ion to silver (metal) on the surfaces of the previously formed nuclei of metallic silver present in axons. The nuclei are thereby enlarged until they are coalescent, and the axons appear as black or brown structures.

Sometimes, the coloration of axons impregnated with silver does not provide adequate contrast under the light microscope. The contrast can be enhanced by adding a third stage of gold toning. To do this, the silver-stained sections are immersed in a solution of gold chloride. If this does not produce the adequate increase in contrast, then it is necessary to add a further stage of reduction in oxalic acid. Finally, the
sections are immersed in aqueous sodium thiosulphate to remove residual silver salts. The increase in contrast is due to a reduction in the unwanted argyrophilia of the background rather than to an intensification of the metallic deposits in the axons (Gruber, 1981).

3.2 Methods

3.2.1 Design and development of the multiple-lumen nerve cuff

Some of the important factors involved in the development of a nerve cuff are:

1. fabrication considerations,
2. chemical and microstructural properties,
3. design considerations, and
4. biological variability considerations.

As mentioned in the literature review, one of the most important factors essential for successful regeneration of axons is providing a clear path, free of dead tissue, blood clots and other debris, for the regenerating axonal growth tips (Lundborg et al., 1981). The design chosen for the experimental multiple-lumen nerve cuffs consisted of two medical grade silicone rubber tubes. The multi-lumen tube was placed coaxially inside a single-lumen SilasticR tube. The SilasticR single-lumen tube was purchased from Dow Corning (Dow Corning Corp., Midland, Michigan). The inner diameter of this tube was 1.58 mm and the outer diameter was 2.41 mm with a wall thickness of 0.41 mm. The 7-lumen silicone rubber tubing (diameter of the 7-lumen tubing = 2.44 mm) was provided by Mr. Charles Heide Jr., Vesta Inc., Greendale, Wisconsin.
The diameter of the 7-lumen tubing was comparable to actual diameters of 1.2 to 1.6 mm of the sciatic nerves in Sprague-Dawley rats (weighing 200-250 gms) at the mid-thigh level. The diameter of each lumen was 0.381 mm. It was decided to keep the wall thickness of the outer single-lumen silicone rubber tube as small as possible and still not compromise the physical integrity of the cuff because the cuff has to be flexible for it to be implanted. Also, thinner walls enabled the sutures to be placed easily through the outer tube.

The single-lumen silicone rubber tube was first cleaned by immersing it in boiling sodium bicarbonate solution. Then the tube was rinsed thoroughly with distilled water and air dried. The cleaned tube was cut into 11 mm long sections using a special tube holder and a new regular industrial razor blade. Initially, cross-sections from the 7-lumen tubing were obtained and examined using an optical microscope to ensure that the diameters of the holes were uniform and that the holes remained open throughout the length of the tube. The 7-lumen tube was also cleaned with boiling sodium bicarbonate, rinsed, air dried, and cut into 5 mm long pieces. Care was taken to ensure that the cut edges were perpendicular to the tube axis and that there were no rough surfaces. The single-lumen tubes were then immersed in xylene for 20 minutes to cause swelling. After the tubes had suitably expanded, the 7-lumen tubes were slipped into the center of the single-lumen tubes, and the whole double-tube cuff was dried in an oven at 70°C for twelve hours to allow the xylene to evaporate. The outer tube by then had shrunk to the diameter of the 7-lumen tube and formed a pressure fit over the 7-lumen tube. The cuffs were then thoroughly washed with distilled water and air dried before storage.
The gap length between the severed nerve ends was 5 mm. Allowing 3 mm on each end for inserting the nerve stumps, the total length of the cuff was 11 mm. The length of the cuff and the gap length were based on the amount of space available at the mid-thigh level of Spague-Dawley rats weighing between 200-250 gms (Lundborg et al., 1982a). A longitudinal section through the double-tube cuff is shown in Figure 3.1.

![Longitudinal section through the middle of the double-tube nerve cuff](image)

**Figure 3.1:** Longitudinal section through the middle of the double-tube nerve cuff

The pattern of holes through the 7-lumen cuff is shown in Figure 3.2. The hole arrangement consisted of one centrally located hole with six other holes arranged in a circle around it. The distance between the central hole and the circularly arranged holes was 0.305 mm.
The protruding ends of the outer tube served as lip-like structures at the ends of the cuff for placing sutures when the nerve ends were inserted into the cuff. The cuffs were usually made a day before surgery was scheduled and stored in distilled water. The cuffs were sterilized by immersing them in 70% ethanol for two hours before surgery. Just before implantation, the cuffs were washed with sterile saline and the lumen was filled with sterile saline.

Figure 3.2: Cross section through the 7-lumen tube showing the hole arrangement

The single-lumen nerve cuffs used in the in vivo studies were made from silicone rubber tubing from Dow Corning (Dow Corning Corp., Midland, Michigan). The inner diameter of this tube was 1.58 mm and the outer diameter was 2.41 mm with a wall thickness of 0.41 mm. The dimensions of these cuffs were identical to
the dimensions of the outer single-lumen silicone rubber tubes used to fabricate the multiple-lumen cuffs. The single-lumen tubes were also cut into 11 mm long sections, and cleaned, rinsed, and sterilized similar to the multiple-lumen cuffs. No xylene treatment was done on the single-lumen cuffs. The total cross-sectional area of all seven lumens in the multiple-lumen silicone rubber cuff was 0.798 mm\(^2\) compared to a total cross-sectional area of 1.961 mm\(^2\) in the single-lumen silicone rubber cuff. The total surface area provided by all the seven lumens in the 11 mm long multiple-lumen cuff was 41.89 mm\(^2\) compared to a total surface area of 24.82 mm\(^2\) in the single-lumen cuff of same length. This represents a 2.5x decrease in total cross-sectional area and a 1.7x increase in total surface area in the multiple-lumen cuffs compared to the single-lumen cuffs of the same length.

3.2.2 In vivo experimentation

The second phase was the in vivo implantation of the prosthesis. Forty four adult male Sprague-Dawley rats weighing between 200 and 250 gms were divided into a total of seven groups (see Table 3.1 for details). Groups I, II, and III consisted of 4, 8, and 16 rats, respectively, and served as controls. Groups IV, V, VI and VII included four rats each, and they were the experimental groups. Animals in groups IV, V, VI and VII were terminated at the end of 8, 12, 16, and 24 weeks post-implantation, respectively. Previous work with single-lumen silicone chambers implanted on both sides in the sciatic nerve of the rat exhibited the same degree of variability as did corresponding chambers implanted in different animals (Danielsen et al., 1988b). Therefore, no advantage was expected in the present study from contrasting the performances in the different repair methods on opposite sides of the
same animal.

Group I was not operated upon. One animal was sacrificed from this group at the end of 8, 12, 16 and 24 week periods. The sections of the sciatic nerve removed from these control animals were subjected to the same tissue processing procedures as the retrieved nerves from the animals having surgery. These control sections were used to compare factors such as number and percentage area occupied by myelinated axons, and organization of regenerated axons with that for the experimental sections for the time periods specified.

Table 3.1: Classification of animals into groups based on type of nerve repair and implant periods

<table>
<thead>
<tr>
<th>Type</th>
<th>Group Number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Implantation Periods&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8 weeks</td>
</tr>
<tr>
<td>Control (no surgery)</td>
<td>I (4)</td>
<td>(1)</td>
</tr>
<tr>
<td>Control (end-to-end repair)</td>
<td>II (8)</td>
<td>(2)</td>
</tr>
<tr>
<td>Control (single lumen cuff)</td>
<td>III (16)</td>
<td>(4)</td>
</tr>
<tr>
<td>8 week exptl.</td>
<td>IV (4)</td>
<td>(4)</td>
</tr>
<tr>
<td>12 week exptl.</td>
<td>V (4)</td>
<td></td>
</tr>
<tr>
<td>16 week exptl.</td>
<td>VI (4)</td>
<td></td>
</tr>
<tr>
<td>24 week exptl.</td>
<td>VII (4)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of rats in each group in brackets.

<sup>b</sup> Number of rats sacrificed is shown in brackets.

3.2.3 Surgical protocol

Anesthesia was induced in all animals with intraperitoneal Nembutal<sup>R</sup> (Abbott Laboratories, Chicago, IL, Lot #26807AF). The dose used was 50 mg/kg body
weight. The required dosage was administered by an intraperitoneal injection into the lower left quadrant of the abdomen of the rat. After the animal had reached the suitable level of anesthesia, the animal was placed on a stainless steel tray in the prone position. The right leg was stretched and the hair was shaved from the entire dorsal surface using a pair of animal hair clippers. The hair was removed from the diaphragm, cranially, to the root of the tail, caudally, and medially to the midline of the vertebrae. Any hair on the ventral side of the right thigh and right leg was also removed. The loose hair was brushed off and the animal was transferred to a clean stainless steel tray and placed in the prone position. The fore limbs were stretched forward cranially and taped to the tray. The head was also immobilized by tape. The right hind limb was stretched caudally such that the trochanter region of the right femur projected on the dorsal side of the right thigh. Both hind limbs were also taped to the tray. The exposed skin area was alternately cleaned with sponges soaked with 70% ethanol and with betadine solution. The scrubbing was started at the incision site, usually near the center of the clipped area. A circular scrubbing motion was used, moving from the center to the periphery of the clipped area. Care was taken not to return a sponge from the periphery to the center to avoid contamination. Sponges were discarded after reaching the periphery. The cleaned area was never touched by ungloved hands, and excess betadine or alcohol was wiped off carefully from the surface of the steel tray. The alcohol and betadine scrubbing was usually repeated three times. The animal was then transferred to the surgery table. A small strip of cotton gauze was placed under the animal’s mandible to keep the head held high and keep the nostrils open and clean. The entire animal, except the head, was covered with a sterile Steri-Drape^R (3M Medical, St. Paul, MN). From
this point onwards, aseptic conditions were maintained at the surgery site.

The skin incision was made using a size 20, sterile rib-back carbon steel blade (Brad-Parker, Rutherford, NJ). The projection of the greater trochanter of the right femur was palpated. The incision was started just caudal to this bony projection and was continued cranio-laterally towards the patella. The skin incision followed the cranial edge of the biceps femoris muscle from the midline proximally towards the patella distally. Once the superficial muscles were exposed, the fascia separating the cranial edge of the biceps femoris and the tensor fascia latae laterally was gently separated using blunt dissection. Similarly, the fascia separating the biceps femoris and gluteus superficialis medially was also separated by blunt dissection. Both muscle groups were spread apart manually and the deeper layers were exposed. The incision was held apart by a Weitlaner self-retaining retractor. A 5 mm section of the sciatic nerve, which runs in the groove between the adductor magnus and quadratus femoris cranially and the semimembranosus caudally, was isolated from the surrounding tissue by blunt dissection. The incision area was always kept moist with a sponge soaked with sterile saline. The sciatic nerve was gently lifted at a spot about 3 to 4 mm proximal to the bifurcation into the common peroneal and the tibial branches and a clean cut was made in one motion with a pair of new, sharp, curved Mayo dissecting scissors. Once the nerve was severed cleanly into a proximal and distal segment, then the procedure varied according to the type of repair technique followed (see Figure 3.3).

In the case of the single-lumen cuff and multiple-lumen cuff groups, the surgical procedure followed in inserting the cuffs was the same. One end of the sterile, saline filled, cuff was spread open by a hemostat, and a needle (3/8 circle, spatula) with a
Figure 3.3: Position of the implanted nerve cuff relative to the rat's thigh (Seckel et al., 1984)

9-0 silk ophthalmic suture (black twisted, diameter range between 0.03mm and 0.039 mm) (Ethicon Inc., Somerville, NJ) was passed through the wall of the cuff from the outside as shown in section a of Figure 3.4. The proximal stump was gently grasped with a 4 inch iris tissue forceps, and the needle was passed through the epineurium about 2 to 3 mm from the cut surface of the nerve as shown in section b of Figure 3.4. Then the needle was passed through the wall of the cuff from the inside as shown in section c of Figure 3.4. Section d in Figure 3.4 shows how both ends of the suture were pulled in together such that the end of the nerve stump slipped into the lip of the cuff. Once the stump was inserted the required depth of 3 mm into the cuff, a square knot was thrown to prevent the cuff from slipping off the end of the nerve stump. Since the knot was placed over the outer wall of the cuff, this did not place any direct tension on the nerve itself. The distal stump was also inserted and sutured similarly.
so that a gap of 5 mm existed between the stumps. Some of the saline inside the
cuffs leaked while maneuvering the cuffs. This saline was replaced by injecting sterile
saline through the wall of the cuff with a hypodermic syringe into the lumen of the
cuff between the stumps. All cuffs used in groups IV, V, VI, and all cuffs in group III,
except the 24 weeks implantation period animals, were filled with sterile saline before
closing the incision site. The cuff was then placed in position in the groove, and the
muscle layers were pulled back into position. A simple continous pattern was used
to suture the biceps femoris to the tensor fascia latae and the gluteus superficialis
using 4-0 chromic gut suture material (Davis and Geck, Danbury, CT). Finally, the
skin was closed with 2-0 DermalonR (Davis and Geck, Montreal, Canada) using a
simple interrupted pattern. Bitter Orange (Arc Laboratories, NY) was applied to
the closed skin incision and the right hind-limb and footpad to avert self-mutilation
by the rat (Uzman and Villegas, 1983).

The animals were observed in the laboratory until they were awake and alert.
They were then returned to the animal care facility for routine postoperative moni­
toring and care. Animals were housed in temperature and humidity controlled rooms
with twelve hours light cycles, and they had access to food and water ad libitum.
A sufficient amount of sawdust was present in each cage to decrease contact of the
paralyzed limb with the hard unyielding floors of the plastic cages. Skin sutures were
removed in 7 to 10 days after surgery.

No cuffs were implanted in the animals in Group II. The sciatic nerves in these
animals were repaired by the end-to-end anastamosis technique. The right sciatic
nerve was exposed at mid-thigh level. At a convenient location, about 2 to 3 mm
proximal to the bifurcation, the sciatic nerve was sharply transected with microscis-
Figure 3.4: Procedure for suturing the transected nerve to the silicone rubber nerve cuff (Seckel et al., 1984)
sors. The nerve ends were inspected to see if a clean cut had been achieved. Neural anastomosis was accomplished by epifascicular epineural coaptation (the epifascicular epineurium is the portion of non-fascicular collagen connective tissue that surrounds the fascicles circumferentially). The epifascicular neural coaptation consisted of the placement of two or three sutures in this layer. The epifascicular epineurium of the proximal nerve segment was carefully grasped with a jeweller's forceps. A 9-0 silk ophthalmic suture (black twisted, diameter range between 0.03 mm and 0.039 mm) (Ethicon Inc., Somerville, NJ) was then passed through the epifascicular epineurium, avoiding injury to the fascicles. Suture placement was approximately two to three needle breadths from the cut margin of the epineurium. After passing the needle through the epineurium, the needle was grasped with a needle holder. The epifascicular epineurium of the distal cut nerve section was then grasped, and the needle was passed through in a similar fashion. Then the suture was tied using a square knot. Suture placement was done with minimal tension. A second suture was then placed approximately 180° opposite to the first suture. Two sutures were used to repair the transected nerve in all the animals in group II except the 24 weeks implantation period animals. In the case of the 24 weeks animals, three sutures were placed at approximately 120° to each other. Two animals from this group were sacrificed at the end of 8, 12, 16, and 24 weeks post-surgery, respectively.

Group III served as controls for single-lumen silicone rubber cuffs. The sciatic nerve was exposed, transected sharply with microscissors, and pulled apart to form a 5 mm gap between the stumps. The stumps were inserted in the proximal and distal ends and held in place by placing one 9-0 silk ophthalmic suture (black twisted, diameter range between 0.03 mm and 0.039 mm) (Ethicon Inc., Somerville, NJ) at
both ends through the epineurium and the wall of the tube (see Figure 3.5).

Figure 3.5: 5 mm gap between the transected proximal and distal stumps bridged by a control single lumen cuff (Seckel et al., 1984)

Two animals from this group were sacrificed at the end of 8, 12, 16, and 24 weeks post-implantation, respectively. The surgical procedures followed for Groups IV, V, VI, and VII were identical to that of Group III except that for these groups, the experimental cuffs were implanted.

The 8, 12, 16, and 24 week time periods were chosen based on some preliminary experiments. A pilot study was conducted in which the multiple-lumen cuff was implanted in the rat sciatic nerve and retrieved at 2, 4, and 8 weeks post-implantation. The 2 and 4 weeks study proved inconclusive. The pilot 8 weeks study showed axonal growth through three of the seven holes in the implanted experimental cuff. Cross sections stained with Hematoxylin and Eosin showed several well defined capillaries. Sections stained with Luxol Fast Blue-Holmes' Silver Nitrate showed very little myelination of the regenerated axons.
3.2.4 Videotape evaluations

The animal's ability to use the right hind leg for walking and to bear weight, and the degree of functional recovery were assessed by videotaping the animal's movements and rating these characteristics against a scale similar to the one used by clinicians to evaluate neurological functional recovery in limbs. Prior to implant retrieval, the animal was placed in a large cardboard box with walls on all sides, and the animal was allowed to get accustomed to its surroundings. A placard with only the corresponding animal number was first videotaped before each animal's movements were also recorded for about five minutes. After all the recordings were made, each animal's movements and the ability to use its right hind leg were evaluated by five observers independently. The evaluations were blind because the evaluators had no knowledge of each animal's group. The rating scale used to evaluate the animals was:

(0) Worst case, lame.

(1) Decreased strength, severely abnormal gait, exaggerated leg movements, loss of motor control in the right leg, severely curled digits.

(2) Some gait abnormalities, leg movements not very exaggerated, some weight-bearing ability, digits less curled.

(3) Normal, used leg well in most aspects.

The ranking assigned by each observer and the remarks made for each animal are listed in Table 4.5 under Results and Discussion.
3.2.5 Electrophysiological evaluations

The Compound Action Potential (CAP) was used to evaluate physiological regeneration across the repaired nerve in vivo. The area under the CAP, which is measured by integration of the Compound Action Potential (called ICAP), gives an indication of the total proximal axons with distal connection.

Just before sacrifice, both control group and experimental group animals were anesthetized with intraperitonial sodium pentobarbital. The anesthetized animal was placed on a wooden board in a prone position and the board was placed on a metal base plate inside a Faraday cage. The Faraday cage minimized 60 Hz interference. The right sciatic nerve was exposed and carefully dissected free of surrounding tissue from the sciatic notch cranially to the bifurcation into the common peroneal and tibial nerves caudally. The repair site was not disturbed. The exposed area was bathed in mineral oil maintained between 26°C and 30°C.

A pair of silver stimulating hook electrodes (made of 26 gauge silver wire) were placed under the nerve, about 1 to 2 mm proximal to the bifurcation. This position was about 2 to 3 mm distal to the repair site and was termed as the distal site. The other set of recordings was made by placing the stimulating electrodes about 2 to 3 mm proximal to the repair site. This position was termed the proximal site. Another pair of identical silver differential recording hook electrodes were placed under the nerve 2 mm from the sciatic notch, proximally. Once set, the position of the recording electrodes was not changed throughout the experiment. The stimulating and recording electrodes were mounted on separate micro-manipulators with magnetic bases. By activating the magnet, the bases were locked into the desired position on the metal base plate. The distance between the stimulating electrodes at the proximal site and
the recording electrodes varied between 3.5 cm and 4.5 cm. The distance between the stimulating electrodes at the distal site and the recording electrodes varied between 5 cm and 6 cm. The same recording and stimulating electrodes were used to make all the measurements in all the animals. Thus, the recording configuration was identical for both stimulation sites. The shielded cable from the stimulating electrode was connected to a Grass SD-9 stimulator, which also triggered a sweep in an oscilloscope. The polarity on the stimulating electrode was set in such a manner that the cathode of the stimulating electrode was nearest to the recording electrode to prevent anode block. A monophasic, square pulse with a duration of 0.1 millisecond was used to deliver a stimulatory current to the nerve. For each animal, and for each stimulating position, the maximum pulse amplitude was set at levels sufficient to stimulate the total population of axons. The proximal stimulating voltages were between 0.5 volts and 2 volts and the distal stimulating voltages were between 5 volts and 20 volts for animals with repaired nerves. The stimulating voltages for normal control animals (group I) were between 0.5 volts and 2 volts.

The recording electrodes were connected to a pre-amplifier which amplified the recorded signal 100 times. Then the amplified signal was fed into one channel of a multi-channel Tektronix® 511A storage oscilloscope. The signal was amplified and filtered with a bandpass of 0.1 Hz to 10 kHz. The output signal was viewed on the oscilloscope screen and stored (see Figure 3.6). The displayed signal was then photographed using a Tektronix® C-4 CRT camera and Polaroid® instant pack film (667 Professional, Polaroid Corp., Cambridge, MA) for permanent records. A manual tracing of the signal was made from the photograph. The Image Analysis Facility in the Department of Veterinary Anatomy, Iowa State University, acquired
images of the tracing using a Zeiss SEM-IPS image analysis system (Zeiss-Kontron, IBAS version 2.00). The tracings were placed on a copy stand and images were captured using a Sony 3 CCD color video camera equipped with a Fujinon Zoom lens. The internal scaling feature of the image analysis software was calibrated for the images under investigation. Calibration was done by viewing a scaling grid (which had one square millimeter grids) at the required magnification and marking off one millimeter distances in the X and Y axes. The software automatically determined the number of pixels that were needed to make up one millimeter at that particular magnification. The outline of each tracing was discriminated. The outline was then filled and measured to obtain the total area under the signal between the outline of the signal and base line on the tracing. The same procedure was followed in acquiring both the proximal and the distal site integrated compound action potential (ICAP) areas in all animals in which the electrophysiological studies were done.

The area of the ICAP measured between the proximal stimulating electrode (i.e., when the stimulating electrode is at the proximal site) and the recording electrode is a measure of the total population of axons in the nerve. The area of the ICAP measured from stimulation at the distal electrode (i.e., when the stimulating electrode is at the distal site) is a measure of the axons that have functionally reconnected across the repair site. Electrical stimulation distal to the repair site evokes an impulse only in those parent fibers that have at least one regenerated branch, and because of mutual occlusion, parent fibers with many regenerated branches will not produce any more impulses than those with only one. The distal ICAP divided by the proximal ICAP is the ratio of the total proximal axons with functional distal connection, normalized to the total proximal axon population.
Figure 3.6: Electrophysiological setup
In some animals, the conduction velocity (CV) was also measured at sites proximal and distal to the repair area. Maximum conduction velocities were calculated by measuring the latency of conduction to the peak of the action potential for measured inter-electrode distances (using calipers under direct vision), using the following formula:

\[
CV(\text{proximal or distal}) = \frac{\text{Distance between cathodes}}{\text{Latency}}
\]

The measurements of the Compound Action Potentials and conduction velocities are subject to several errors inherent in the measuring techniques. The conduction velocities measured through the repair segments can be underestimated by 5% depending on the distances of the stimulating electrodes from the repair site and the relative conduction velocities in the proximal and regenerated segments of the nerve. Efforts were made to minimize other sources of error such as measuring the inter-electrode distances, temperature fluctuations, degradation of preparations, and damage to delicate regenerated structures. The conduction velocity method is subject to many assumptions regarding the current path through the whole nerve and the spread of depolarization. This is because the contributions of resistance and capacitance of endoneural, perineural, and epineural components may be different for nerves in different stages of regeneration (Fields and Ellisman, 1986a). For these reasons, our methods may not have been sensitive enough to detect small but significant differences between some measurements or to provide a quantitative comparison between different groups of animals.
3.2.6 Gross visual examinations

The initial gross evaluations were made on the right hind leg before sacrifice. Plantar ulcerations, if present, were noted. The number of digits that were missing due to self-mutilation was also noted. The second evaluation was made when each animal was biopsied. The factors assessed were nerve adhesion to surrounding tissue, presence of neuromas, and gross nerve alignment. Nerve adhesion was rated against a relative scale from minimum to severe, with three intervals in between, minimum/moderate, moderate, and moderate/severe, respectively. Finally, a low magnification (between 10x and 30x) visual examination was done on the retrieved and formalin fixed repair sites using a dissecting microscope. The number of regenerated tissue strands bridging the stumps inside the lumen of the multiple-lumen cuffs were counted. The shape and position of the connecting tissue bridge was noted in the single-lumen cuffs. None of the gross evaluations were blind. See Table 4.1 under Results and Discussion for comparative data.

3.2.7 Histology

3.2.7.1 Fixation After the in vivo electrophysiological studies were completed at the repair site, a 15 mm to 17 mm section of the right sciatic nerve including the repair site and 2 mm to 3 mm of the stumps on each side was removed. The proximal stump of the nerve was cut about 2 mm to 3 mm proximal to the repair site. The distal stump was cut about 2 mm to 3 mm distal to the repair site. The retrieved nerve was immediately immersed in a 10 % neutral buffered formalin (NBF) preservative. The volume of the NBF used for fixation was at least ten times that of the nerve tissue. The NBF was changed after the first 24 hours. After fixation, the
repair site was trimmed to remove the fibrous tissue encapsulation around the cuffs and the non-neural tissue layer around the neuroma in the end-to-end repairs. The outer silicone rubber tube of the multiple-lumen cuffs was then carefully cut away using a scalpel blade, thereby exposing the 7-lumen inner silicone rubber tube with the connecting tissue strands inside the lumens and the attached stumps on both sides. A fresh scalpel blade was then used to cut the connecting tissue strands distally, precisely at the junction between the strands and the distal stump. The proximal stump was grasped and the tissue strands (which were still attached to the proximal stump) were very carefully pulled out of the lumen of the multiple-lumen cuff, thereby exposing them fully. The silicone rubber cuff was also sliced and removed to expose the central connective bridge of tissue in the single-lumen cuffs.

Three sections, about 2 mm to 3 mm long, were removed from the proximal, middle, and distal regions of the repair area for further histological processing. It must be noted here that the increased cellular and fibrous tissue reactions, both between the ends of the tubes and the surfaces of the inserted stumps, and also between the outer tube wall and surrounding tissues, must be far enough removed from the regions selected for observation and study to allow uncluttered observation and evaluations of the regenerated nerve tissue. Also, for purposes of quantitative analysis, some standard sampling regions were needed that could be located precisely in all the repair sites. Based on these factors, in the multiple-lumen cuffs, the proximal section was removed, 4.5 mm proximal to the center of the cuff, the middle section from the center of the cuff, and the distal section was removed, 4.5 mm distal to the center of the cuff. In the case of the single-lumen cuffs, the proximal section was removed, 4 mm proximal to the center of the cuff, the middle section from the center
of the cuff, and the distal section was removed, 4 mm distal to the center of the cuff. For the end-to-end repair, the proximal and distal sections were removed similarly, 4 mm proximal and distal to the center of the repair site, and the middle section was removed from the center of the repair site. The 2 to 3 mm sections were centered at these sites. In the normal controls, a 5 mm section of the sciatic nerve corresponding to the repair areas in the other groups was removed for histological processing. Each of the three sections from every repair site was placed in separate, labeled specimen bottles filled with 10% neutral buffered formalin. The sections were processed at the Histo-pathology laboratory in the Department of Veterinary Pathology, Iowa State University.

3.2.7.2 Dehydration After fixation, the sections were dehydrated in ascending concentrations of ethanol in the following order:

1. 70 % ethanol for 1 hour
2. 80 % ethanol for 1 hour
3. 95 % ethanol for 1 hour
4. 100 % ethanol for 1 hour
5. 100 % ethanol for 1 hour
6. 100 % ethanol for 1 hour.

3.2.7.3 Embedding After dehydration, the sections were embedded using the JB-4^R embedding kit from Polysciences Inc. (Polysciences Inc., Warrington,
PA). Tissue embedded in JB-4 embedding medium offers superior ultrastructural preservation and semi-thin sections for high resolution light microscopy. This water-soluble plastic medium also provides higher clarity and contrast than paraffin, and offers the advantage of less distortion and tissue shrinkage. Other advantages are extremely fast processing times, not having to completely dehydrate the tissue before infiltrating because the plastic is water-soluble, and not having to use clearing agents such as xylene, benzene, chloroform, or toluene.

The embedding was done in two stages, namely infiltration and polymerization.

**Infiltration** The JB-4 embedding kit consisted of a catalyst, solution-A, and solution-B. The catalyzed infiltration resin was made by adding 0.90 gms of dry catalyst to 100 ml of solution-A and mixing well until dissolved. The tissue sections were placed in the resin and maintained at 4° C. After two hours, the tissue sections were transferred to another freshly made resin infiltrating medium for twelve hours at 4° C.

**Polymerization** The embedding medium was made by adding 1 ml of solution-B to 25 ml of freshly prepared catalyzed solution-A, stirring well and placing in an ice-bath while embedding. Initially, only about a third of the mold was filled with the embedding medium, and the tissue was positioned in the viscous resin at the required orientation. The mold was then completely filled and capped tightly. Since anaerobic conditions were necessary for polymerization, the caps were sealed with paraffin around the edges. Polymerization was completed by storing the sealed molds in a vacuum dessicator at 4° C for twelve hours.
3.2.7.4 Sectioning  The polymerized blocks were exposed to air for a few hours before sectioning because blocks cut easier after the exposure. Semi-thin cross-sections of the nerve, between 1.5 μm and 2.5 μm thick, were cut with a dry glass knife on an ultra-microtome, collected with forceps, and transferred onto a room-temperature water bath surface. The sections were released before they touched the water. The sections were then collected on pre-cleaned glass slides and air-dried before staining.

3.2.7.5 Etching and staining  The semi-thin sections had to be etched to remove the plastic matrix prior to staining.

**Etching**  A saturated solution of sodium hydroxide in 100% ethanol was made 24 hours prior to the etching procedure. Just before etching, this solution was diluted to make a 10% solution of sodium hydroxide in 100% ethanol. One or two drops of the etch solution was placed over the sections for 30 minutes to etch away the plastic matrix. The slides were then washed with a few drops of 100% ethanol and dried.

**Bodian staining**  This method utilizes a silver proteinate compound to impregnate neural tissue sections. Protargol-S, which is the name usually used to describe the silver proteinate, is made from partially hydrolyzed protein. The tissue sections are first treated in the Protargol-S solution to which metallic copper has been added. The neural and connective tissue are the first to be impregnated. The addition of copper reduces the amount of silver taken up by connective tissue elements because the copper is more reactive than silver and displaces it from the connective tissue. Thus, the copper greatly enhances the contrast between the neural and connective tissue elements. The subsequent treatment with hydroquinone reduces the
silver deposited on the neural elements to visible, opaque, metallic silver. The gold chloride and oxalic acid treatments act as stain intensifiers, and the final sodium thiosulfate treatment fixes the silver in the tissue by stopping all previous reactions and removing unreduced silver salts.

Preparation of staining and reducing solutions

- **Staining solution** 2% Protargol-S. 8 to 12 gms of bright, shiny metallic copper shot (previously cleaned with an acid mixture containing 3 parts hydrochloric acid to one part nitric acid for 5 minutes until the dark surface oxide film layers were removed) was placed at the bottom of a chemically cleaned glass beaker containing 100 ml of double distilled water. 2 gms of Protargol-S (Polysciences Inc., Warrington, PA) was sprinkled on top of the water and allowed to dissolve from the surface downward, without stirring.

- **Reducing solution** 1.0 gm hydroquinone and 5.0 ml of 40% formaldehyde were added to 100 ml of double distilled water in an acid-cleaned glass beaker.

Staining schedule

1. Placed slides in freshly prepared Protargol-S solution for 48 hours in a 37°C oven.

2. Washed very briefly in double distilled water.

3. Reduced in freshly prepared reducing solution for 10 minutes.

4. Washed thoroughly in double distilled water to remove all of the reducing solution.
5. Toned in 0.1% solution of gold chloride for 10 minutes.

6. Rinsed in double distilled water.

7. Placed in 2% oxalic acid for 10 minutes or until sections had a faint but definite purplish or bluish tinge.

8. Rinsed in double distilled water.

9. Treated in 5% sodium thiosulfate for 10 minutes.

10. Washed thoroughly in tap water.

11. Dehydrated in 95% and absolute ethanol, cleared in xylene; two changes each.


The Bodian stain stained both the myelinated and unmyelinated axons black or purplish black. The myelin sheaths did not stain. This left clear, unstained, circular rings around the myelinated axon cores. The background was violet to dark red with some of the endoneural connective tissue also staining violet (due to excess staining). Blood vessels and erythrocytes were also stained pink to dark red.

3.2.8 Quantitative evaluations

3.2.8.1 Automated image analysis Morphometric parameters of peripheral nerve repair such as axon core diameters, total axonal cross-sectional areas, total regenerated nerve cross-sectional areas, and axon counts were evaluated by an automated image analysis program from the proximal, middle, and distal cross-sections for each animal. Images of the nerve sections were acquired by the Image Analysis
Facility, in the Department of Veterinary Anatomy, Iowa State University, using a Zeiss SEM-IPS image analysis system (Zeiss-Kontron; IBAS version 2.00). Two programs were run, one for measurements of axons at a higher magnification, and the other for the measurements of the regenerated nerve at a lower magnification. The silver stained cross-sections were viewed with a Zeiss Axiophot microscope at 100x magnification (40x by 2.5x optivar). Images were captured from the slides with a Sony 3CCD color video camera. The internal scaling feature of the image analysis software was calibrated to measure in microns. Calibration was done by viewing a precision stage micrometer slide (which had one square micrometer grids) at the required magnification and marking off one micrometer distances in the X and Y axes. The software automatically determined the number of pixels that were needed to make up one micrometer at that particular magnification. Each image that was projected onto the television screen was only a fraction of the entire cross-section of the nerve. After the required measurements were made from each image, the tissue section was moved to obtain the image from a different area of the cross-section. Each image viewed was termed a field and data from several fields were collected until the entire tissue cross-section was scanned. It took between 3 and 5 fields to scan the entire cross-section depending on the size of the cross-section. The image from each field was normalized by spreading the gray levels from the image over the entire range available and the axons were automatically discriminated. Images of the axons were interactively edited when necessary due to staining and contrast variability. Any axons that were not fully in the image screen and axons that were not perpendicular to the plane of the cross-section (having oblique or long wavy shapes) were discarded. A reference frame was drawn around the edges of the image screen. Total tissue area
(the area of the reference frame), sum total of the cross-sectional areas of all the axons that were seen in the field, the cross-sectional area and core diameter of each individual axon, and the number of axons were measured and recorded for each field. The field data from each cross-section was saved in a file with the corresponding animal identification number.

A second program was run for the measurement of the cross-sectional area of the entire regenerated nerve at the proximal, middle, and distal regions. The stained sections were viewed with a Zeiss Axiophot microscope at 3.125x magnification (2.5x by 1.25x optivar). Images were captured from the slide with a Sony 3CCD color video camera. The internal scaling feature of the image analysis software was calibrated to measure in microns as explained before. The cross-sections were discriminated from the captured images and interactively edited. Total cross-sectional area of the entire nerve was measured and recorded. The data from each section were saved with the corresponding animal identification number. All the image analysis data was stored on floppy disks for further calculations and evaluations. See Table 4.3 and Table 4.2 under Results and Discussion for the listing of the data.

3.2.8.2 Preparation of fiber diameter histograms During nerve regeneration, axons ending in scar tissue and branched axons develop at a repair site. Axons ending in scar tissue are blocked, and hence are non-functioning. Furthermore, axonal scarring and blockage stimulates compensatory axonal branching into empty endoneural tubes distal to the repair site. As the demand on the proximal axons increases during this blocking and branching process, the average size of the distal fibers decreases. Because of this, a comparison of the number of myelinated
axons proximal and distal to a repaired nerve site gives an inaccurate estimate of the percentage of connected axons. The use of a fiber diameter histogram (FDH) of a nerve solves this problem, because a FDH measures both the size of the regenerated axons (which is proportional to their maturation) and their number. The data from the automated image analysis was used to generate the fiber diameter histograms and to calculate the mean axon core diameters. The axon core diameter data corresponding to each animal was transferred to a Lotus 1-2-3 spreadsheet (Lotus 1-2-3 Release 2.2, Cambridge, MA) and the mean axon core diameters and axon distributions were generated for the proximal, middle, and distal regions. A schematic of the process of data collection and the generation of the fiber diameter histograms is shown in Figure 3.7. The axon distributions were used to create the fiber diameter histograms (see Appendix A). The mean axon core diameters, mean axon counts, mean area of axons, and mean area of the entire nerve at the three regions for all the groups were also calculated (see Table 4.2 and Table 4.3 under Results and Discussion and Appendix B).

3.2.9 Statistical methods

Two statistical tests were done on the quantitative data obtained from the automated image analysis, the videotaped rankings, and the compound action potential percentages. The first test performed on all the quantitative data was the two-way analysis of variance test, in which the total variation of the data was partitioned into three components attributed, respectively, to the various repair methods (or treatments), the four time periods, and the experimental error, or chance. The analysis of variance table was generated and tested at the 0.05 level of significance to determine
Figure 3.7: Schematic showing the procedure for obtaining the fiber diameter histogram from the distal stump (Marshall et al., 1989)
whether the differences among the means obtained from the treatments were significant, and also whether the differences among the means obtained for all the four time periods were significant. The second test performed was the two-sample t-test comparing differences between means for both the different treatments and different time periods. The analysis of variance test determined if there was a significant difference between treatments or between times. The two-sample t-test determined which of the treatments or times was significant with respect to the others at the 0.05 level of significance (α=0.05). All statistical tests were run using SAS (SAS Release 6.06, SAS Institute Inc., Cary, NC) on the HDS AS/9180 (Wylbur) running under the MVS/XA operating system, at the Durham Computation Center, Iowa State University.
4. RESULTS AND DISCUSSION

4.1 Results

4.1.1 Qualitative results

4.1.1.1 8 weeks
Upon retrieval, it was seen that the host tissue response to four of the implanted cuffs was minimal, and the response to two of the cuffs was minimal to moderate. A fibrous tissue layer encapsulated all the cuffs and the part of the stumps that was adjacent to the cuffs. The response was minimal to one and moderate to the other of the two end-to-end repairs. In group IV, one animal died prematurely, and the transected nerve failed to regenerate in two other animals. A 3 mm long proximal strand was seen inside the lumen in the animal that died prematurely. In the same group, one tissue strand was seen inside the lumen of the multiple-lumen cuff, bridging both stumps in the fourth animal. One animal in group III died prematurely, and a central tissue bridge was seen inside all four of the single-lumen cuffs. The mean diameter of the regenerated cable was smaller at the middle of the single-lumen cuff and progressively increased towards the stumps. Fusiform shaped neuromas were seen in both end-to-end repair animals.

4.1.1.2 12 weeks
Adhesion was minimal in two and moderate in five of the cuffs. Fibrous tissue encapsulation was noted in all the cuffs in both groups III, and
V. Two of the multi-lumen cuffs in group V had dislodged and the transected nerve failed to regenerate through the cuff. Two other animals in group V had three and seven tissue strands inside the multi-lumen cuffs, bridging the stumps, respectively. See Figure 4.1 and Figure 4.2 for a macroscopic view of the retrieved multi-lumen cuff repair sites with and without the silicone rubber tubes. In group III, one animal died prematurely, and a centrally tapering tissue bridge was seen inside the lumen of the cuffs of the other three animals. No neuromas were seen at the suture site in the end-to-end repairs.

4.1.1.3 16 weeks Two of the cuffs in groups III and VI had minimal adhesion, four had moderate adhesion, and in two others, the adhesion was severe. All cuffs were encapsulated by a fibrous tissue sheath. In group VI, two cuffs had five bridging tissue strands, one cuff had six bridging tissue strands, and the fourth cuff had all seven lumen filled by bridging tissue strands, connecting both stumps. Also, in three out of four animals in group VI, the proximal stump of the sciatic nerve had branched a few centimeters proximal to the cuff, and the branch grew distally, circumventing the repair area. All four single-lumen cuffs in group III had a centrally tapered bridge of tissue inside. Fusiform shaped neuromas were seen in both of the end-to-end repairs.

4.1.1.4 24 weeks In groups III and VII, minimal adhesion was seen in two animals, minimal to moderate adhesion was noted in two animals, moderate adhesion in three animals, and one case of severe adhesion in the eighth animal. One of the multi-lumen cuffs in group VII had dislodged, and the proximal stump failed to regenerate through the cuff. One animal in the same group had two bridging tissue
strands inside the cuff, and a second animal had five strands bridging both stumps and a sixth strand extending half-way across the cuff from the proximal stump. Another animal had five bridging strands inside the cuff. In group III, all four animals had a centrally tapered tissue bridge inside the single-lumen cuffs. Group II had fusiform shaped neuromas at the repair sites. See Figure 4.3 for a macroscopic view of a single-lumen cuff repair site after retrieval and fixation. Note the tapering of the diameter in the center of the regenerated tissue bridge. See Figure 4.4 for a macroscopic view of the repair site from an end-to-end repair site. Note the neuroma formation at the middle of the repair region at the suture line.

In summary, in all the multiple-lumen groups for all four time periods, the cuff was dislodged in three cases, the cuff was in position, but empty in two cases, and one animal died prematurely. It must be noted that in two of the three cases where the cuff got dislodged, a non-neural tissue cable grew outside the cuff and connected the stumps. For the single-lumen cases, in all the four time periods, two of the animals died prematurely. Among the group II animals, six of the animals had neuromas at the suture site, and two animals did not have any neuromas at all. All animals implanted with cuffs had a fibrous tissue sheath around the cuffs. The extent of adhesion of surrounding tissue to the repair site increased in the 16 and 24 weeks time periods. See Table 4.1 for a listing of the results.

4.1.2 Histology

The sciatic nerve in the rat is a typical mammalian nerve. The cellular components of the nerve are myelinated and unmyelinated axons, Schwann cells, capillaries, fibroblasts, and occasional mono-nuclear reticuloendothelial cells.
Figure 4.1: Macroscopic view of the multiple-lumen cuff repair site after retrieval and fixation. Animal #11, group VI, 16 weeks post-implantation. Scale bar = 1 mm

Figure 4.2: Macroscopic view of the tissue strands from inside the lumen of the multiple-lumen cuff after retrieval and fixation. Animal #11, group VI, 16 weeks post-implantation. Scale bar = 1 mm
Figure 4.3:  Macroscopic view of single-lumen cuff repair site after retrieval, fixation, and removal of the silicone rubber tube. Animal # 5, group III, 24 weeks post-implantation. Scale bar = 1 mm

Figure 4.4:  Macroscopic view of an end-to-end repair site after retrieval and fixation. Animal # 18, group II, 16 weeks post-implantation. Scale bar = 1 mm
### Table 4.1: Gross visual evaluations and quantitative electrophysiological results for all groups

<table>
<thead>
<tr>
<th>Animal Type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Implant Period, Number of Weeks (days)</th>
<th>Gross Visual Examinations&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Quantitative Electrophysiology</th>
<th>ICAP&lt;sup&gt;c&lt;/sup&gt; Ratio</th>
<th>Proximal Conduction Velocity (m/s)</th>
<th>Distal Conduction Velocity (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ML</td>
<td>(12)</td>
<td>Minimum Adhesion</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>ML</td>
<td>8</td>
<td>Minimum Adhesion</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>ML</td>
<td>8</td>
<td>Minimum Adhesion</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>ML</td>
<td>8</td>
<td>Minimum/Moderate Adhesion</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>SL</td>
<td>(34)</td>
<td>Minimum/Moderate Adhesion</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>SL</td>
<td>8</td>
<td>Minimum Adhesion</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>EE</td>
<td>8</td>
<td>Moderate Adhesion</td>
<td>N/A</td>
<td>N/A</td>
<td>37.5</td>
<td>37.5</td>
</tr>
<tr>
<td>EE</td>
<td>8</td>
<td>Moderate Adhesion</td>
<td>N/A</td>
<td>N/A</td>
<td>40.2</td>
<td>37.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>ML=Multiple-lumen cuff; SL=Single-lumen cuff; EE=End-to-end repair; NC=Normal control.

<sup>b</sup>Evaluations done at time of implant retrieval on right hind-leg.

<sup>c</sup>ICAP=Integrated Compound Action Potential.

<sup>d</sup>Not available.
<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Type of Repair</th>
<th>Implant Period, Weeks or (days)</th>
<th>Gross Visual Examinations</th>
<th>Quantitative Electrophysiology</th>
<th>ICAP Conduction Velocity</th>
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<td></td>
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<td></td>
<td>Adhesion</td>
<td>Observations</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>EE</td>
<td>8</td>
<td>Minimum Fusiform shaped neuroma at suture site.</td>
<td>130.1</td>
<td>N/A N/A</td>
</tr>
<tr>
<td>39</td>
<td>NC</td>
<td>8</td>
<td>Nil Normal nerve</td>
<td>114.6 45.8</td>
<td>36.4</td>
</tr>
<tr>
<td>21</td>
<td>ML</td>
<td>12</td>
<td>Moderate Cuff dislodged from stumps, connecting tissue bridge outside cuff, cuff empty.</td>
<td>N/A N/A N/A</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>ML</td>
<td>12</td>
<td>Minimum 3 bridging strands inside cuff.</td>
<td>41.5 N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>23</td>
<td>ML</td>
<td>12</td>
<td>Moderate Cuff dislodged from stumps, cuff empty.</td>
<td>N/A 28.0</td>
<td>N/A</td>
</tr>
<tr>
<td>24</td>
<td>ML</td>
<td>12</td>
<td>Moderate 2 most distal digits missing, 7 bridging strands inside cuff.</td>
<td>48.4 29.7</td>
<td>N/A</td>
</tr>
<tr>
<td>25</td>
<td>SL</td>
<td>12</td>
<td>Moderate Central bridge inside cuff.</td>
<td>41.7 21.9</td>
<td>51.4</td>
</tr>
<tr>
<td>26</td>
<td>SL</td>
<td>12</td>
<td>Moderate Plantar ulceration, slight bleeding, 3 most distal digits missing, central bridge inside cuff.</td>
<td>26.9 15.4</td>
<td>62.5</td>
</tr>
<tr>
<td>43</td>
<td>SL</td>
<td>12</td>
<td>Minimum Central bridge inside cuff.</td>
<td>118.2 60.0</td>
<td>62.5</td>
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<tr>
<td>Animal Type</td>
<td>Implant Period, Number of Weeks</td>
<td>Gross Visual Examinations</td>
<td>Quantitative Electrophysiology</td>
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<td>Adhesion</td>
<td></td>
<td>ICAP Ratio</td>
<td>Conduction Velocity</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
<td>(m/s)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Proximal (m/s)</td>
<td>Distal (m/s)</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>SL</td>
<td>Animal died day after surgery.</td>
<td>108.0</td>
<td>26.9</td>
<td>N/A</td>
</tr>
<tr>
<td>27</td>
<td>EE</td>
<td>Moderate No neuroma at suture site.</td>
<td>27.7</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>28</td>
<td>EE</td>
<td>Minimum/Moderate 2 most distal digits missing, no neuroma.</td>
<td>111.9</td>
<td>33.3</td>
<td>30</td>
</tr>
<tr>
<td>29</td>
<td>NC</td>
<td>Nil Normal nerve</td>
<td>69.1</td>
<td>33.3</td>
<td>N/A</td>
</tr>
<tr>
<td>11</td>
<td>ML</td>
<td>Minimum Nerve branched proximal to cuff, 6 bridging strands inside cuff.</td>
<td>88.7</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>12</td>
<td>ML</td>
<td>Moderate Nerve branched proximal to cuff, 5 bridging strands inside cuff.</td>
<td>70.8</td>
<td>34.4</td>
<td>N/A</td>
</tr>
<tr>
<td>13</td>
<td>ML</td>
<td>Moderate Most distal digit missing, 5 bridging strands inside cuff.</td>
<td>140.6</td>
<td>23.4</td>
<td>N/A</td>
</tr>
<tr>
<td>14</td>
<td>ML</td>
<td>Moderate 2 most distal digits missing, proximal stump branched proximal to cuff, 7 bridging strands inside cuff.</td>
<td>96.4</td>
<td>26.8</td>
<td>25.0</td>
</tr>
<tr>
<td>15</td>
<td>SL</td>
<td>Minimum 2 most distal digits missing, central bridge inside cuff.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Animal Number</td>
<td>Type of Repair</td>
<td>Implant Period, Weeks or (days)</td>
<td>Adhesion</td>
<td>Gross Visual Observations</td>
<td>Quantitative Electrophysiology</td>
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<td>-------------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ICAP Ratio</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>16</td>
<td>SL</td>
<td>16</td>
<td>Moderate</td>
<td>3 most distal digits missing, central bridge inside cuff.</td>
<td>76.6</td>
</tr>
<tr>
<td>45</td>
<td>SL</td>
<td>16</td>
<td>Severe</td>
<td>Central bridge inside cuff.</td>
<td>70.9</td>
</tr>
<tr>
<td>46</td>
<td>SL</td>
<td>16</td>
<td>Severe</td>
<td>Central bridge inside cuff.</td>
<td>52.2</td>
</tr>
<tr>
<td>17</td>
<td>EE</td>
<td>16</td>
<td>Minimum</td>
<td>Fusiform shaped neuroma at suture site.</td>
<td>50.2</td>
</tr>
<tr>
<td>18</td>
<td>EE</td>
<td>16</td>
<td>Moderate</td>
<td>Fusiform shaped neuroma at suture site.</td>
<td>96.0</td>
</tr>
<tr>
<td>19</td>
<td>NC</td>
<td>16</td>
<td>Nil</td>
<td>Normal nerve</td>
<td>58.0</td>
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<tr>
<td>1</td>
<td>ML</td>
<td>24</td>
<td>Severe</td>
<td>2 bridging strands inside cuff.</td>
<td>100.3</td>
</tr>
<tr>
<td>2</td>
<td>ML</td>
<td>24</td>
<td>Minimum</td>
<td>5 bridging strands inside cuff, a sixth proximal strand extended half way across cuff.</td>
<td>41.0</td>
</tr>
<tr>
<td>3</td>
<td>ML</td>
<td>24</td>
<td>Minimum/Moderate</td>
<td>2 most distal digits missing, 5 bridging strands inside cuff.</td>
<td>72.7</td>
</tr>
</tbody>
</table>
Table 4.1 (Continued)

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Type of Repair</th>
<th>Implant Period, Weeks or (days)</th>
<th>Gross Visual Examinations</th>
<th>Quantitative Electrophysiology</th>
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<td></td>
<td>ICAP Ratio</td>
</tr>
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<td></td>
<td>Adhesion</td>
<td>Observations</td>
</tr>
<tr>
<td>4</td>
<td>ML</td>
<td>24</td>
<td>Moderate</td>
<td>3 most distal digits missing, cuff dislodged, bridging cable outside cuff, cuff empty.</td>
</tr>
<tr>
<td>5</td>
<td>SL</td>
<td>24</td>
<td>Minimum/Moderate</td>
<td>2 most distal digits missing, central bridge inside cuff.</td>
</tr>
<tr>
<td>6</td>
<td>SL</td>
<td>24</td>
<td>Moderate</td>
<td>Central bridge inside cuff.</td>
</tr>
<tr>
<td>47</td>
<td>SL</td>
<td>24</td>
<td>Moderate</td>
<td>Central bridge inside cuff.</td>
</tr>
<tr>
<td>48</td>
<td>SL</td>
<td>24</td>
<td>Minimum</td>
<td>Central bridge inside cuff.</td>
</tr>
<tr>
<td>7</td>
<td>EE</td>
<td>24</td>
<td>Moderate</td>
<td>Fusiform shaped neuroma at site of suture.</td>
</tr>
<tr>
<td>8</td>
<td>EE</td>
<td>24</td>
<td>Moderate</td>
<td>Fusiform shaped neuroma at site of suture.</td>
</tr>
<tr>
<td>9</td>
<td>NC</td>
<td>24</td>
<td>Nil</td>
<td>Normal nerve</td>
</tr>
</tbody>
</table>
The cellular components are embedded in a collagenous extra-cellular matrix, called the endoneurium. Surrounding the cellular components and the endoneurium is the perineurium, which consists of several sleeves, each of which is made of squamous cells. The epineurium, which surrounds the perineurium, is connective tissue that holds the nerve in place and blends with the general connective tissue of the body. See Figure 4.7 A for a cross sectional view of the sciatic nerve from a normal control rat. This general histologic structure of the normal nerve is altered when the nerve is transected and when the nerve regenerates through a nerve bridge. The histology of the proximal, middle, and distal sections for the different repair methods and the four time periods tested is reported here separately.

4.1.2.1 Proximal section  The histologic structure of the proximal stump for all the repair methods and for all four time periods was similar to that of the normal nerve as described above, except for differences in numbers and sizes of axons, and for an increase in the number and size of blood vessels. Also, the general cross-sectional shape of the repaired nerve was circular, in contrast to the flattened oval cross-sections of the normal controls.

4.1.2.2 Middle section  Significant histologic structural differences were evident at the middle of the repair sites in all the repair methods as compared to the normal nerve. Two zones were evident in the cross-sections obtained from the central bridge of tissue inside the single-lumen cuffs, and from each of the bridging tissue strands inside the multiple-lumen cuffs. The first, a peripheral zone, that contained many blood vessels and consisted primarily of collagenous connective tissue, and the second, a central zone, which contained the neural elements. The peripheral zone
was the regenerated epineurium, and consisted mainly of a collagenous connective tissue matrix, dispersed with numerous blood vessels, and lined on the inside primarily by circumferential cells resembling perineural cells and fibroblasts. In some of the multiple-lumen cuff repairs, the outer zone constituted roughly a third of the entire diameter of the regenerated nerve. The endoneural central zone of the nerve, which was surrounded by the circumferential perineural cells, contained the regenerated nerve proper. This zone contained the regenerated axons, blood vessels, and fibroblasts, all of which were dispersed in a collagenous endoneural connective tissue matrix. A difference distinguishing the central zone of the regenerated nerves, from the normal controls, was the subdivision of the entire central zone into several small fascicles by strands of fascicular perineural cells. The term, fascicular perineural cells was used here to differentiate these cells in the central zone from the circumferential perineural cells that surrounded the central zone and underlay the outer epineural zone. This fasciculation was more prominent in the single-lumen cuff repairs and not as many fascicles were seen in the multiple-lumen cuff repairs. See Figure 4.5 for a sequence of cross sectional views from a multiple-lumen cuff repair site obtained from the proximal, middle, and distal sections.

The middle sections from the end-to-end repairs closely resembled the histologic structure of the normal controls, except for the numbers and size of the regenerated axons and blood vessels. The two zone pattern was not evident in these cross-sections, and several regions consisting of wavy parallel lines over short distances were seen.
Figure 4.5: Light micrographs from the proximal (A), middle (B), and distal (C) sections of a multiple-lumen cuff repair. Animal # 14, group VI, 16 weeks post-implantation. Bodian stain. Scale bar = 50 μm
These wavy lines were longitudinal sections of axons whose axes were not perpendicular to the plane of sectioning. This indicated crossing over of axons from one region to another region of the cross-section. This crossing over was not seen in either the single-lumen cuff repairs or the multiple-lumen cuff repairs.

As in the case of the proximal sections, the general cross-sectional shape of the regenerated nerve was circular for both the cuff repair methods. The cylindrical shape was not imposed on the regenerating nerves by the nerve cuffs in the single-lumen cuff repairs because for all these cases, the regenerated nerves floated free in the lumen of the single-lumen cuffs and there was considerable fluid filled space between the regenerated nerve cable and the luminal wall of the cuff. However, this was not the case in the multiple-lumen cuff repairs, and the regenerated tissue strands filled the lumen of the multiple-lumen cuffs almost entirely. The end-to-end repairs had more irregular shapes because of the neuroma formation at the repair sites.

4.1.2.3 Distal section The histologic structure of the distal section in the single-lumen cuff repairs was similar to that seen in the middle sections for the single-lumen cuff repairs. The two zones were evident with numerous blood vessels in the peripheral region and the central neural region containing the neural elements. However, the distal section of the multiple-lumen cuff repairs were markedly different from that of both single-lumen cuff repairs, and end-to-end repairs. Instead of one circular structure with the two zones, as in the case of the single-lumen repairs, several such circular structures, called nerve bundles here for purposes of discussion, were seen in the multiple-lumen cuff repairs. The number of such nerve bundles depended on the number of tissue strands that successfully regenerated across the
multiple-lumen cuff. The nerve bundles were embedded in a collagenous connective tissue matrix. The arrangements of these nerve bundles in the distal connective tissue matrix coincided with the pattern of holes in the multiple-lumen cuff which were infiltrated by the bridging tissue strands. Thus, a pattern or orientation had already been established for the regenerating nerve fibers before they reached the distal stump. This pattern or orientation was influenced by the arrangement of the regenerated tissue strands inside the multiple-lumen cuff. The histologic structure in each of these nerve bundles was similar to that seen in the middle sections obtained from the tissue strands inside the multiple-lumen cuffs. The peripheral and central zones were present, with numerous blood vessels, and regenerated axons being the most prominent features in the peripheral and central zones, respectively. The central endoneural zone was also fasciculated by strands of fascicular perineural cells, similar to that seen in the middle sections. See Figure 4.6 for a comparison of distal sections obtained from the three repair methods at the 24 weeks post-implantation period. See Figure 4.7 B for a cross sectional view of the distal section of a multiple-lumen cuff repair site.

The histologic structure of the distal section for the end-to-end repairs was similar to that observed in the middle sections for the end-to-end repairs. As mentioned before, several regions showing evidence of crossing over of axons were also observed in the distal sections. The general cross-sectional shape in the distal sections of all the repairs was circular, similar to what was seen in the proximal sections, but in contrast to the flattened oval cross-sections seen in the normal nerve with one pole being slightly rounder and larger than the other.
Figure 4.6: Light micrographs from the distal sections of a multiple-lumen cuff repair (A), (animal # 2, group VII); single-lumen cuff repair (B), (animal # 6, group III); and end-to-end repair (C), (animal # 7, group II). 24 weeks post-implantation. Bodian stain. Scale bar = 50 µm
Figure 4.7: A. Light micrograph of a cross-section of the right sciatic nerve at the mid-thigh level from a normal control, (animal # 9, group I). 24 weeks. Bodian stain. Scale bar = 50 \mu m. B. Light micrograph from the middle section of a multiple-lumen cuff repair, (animal # 11, group VI). 16 weeks post-implantation. Bodian stain. Scale bar = 20 \mu m
4.1.3 Fiber diameter histograms

The fiber diameter histograms (or axon distributions) for the four time periods and for the repair methods are displayed in Appendix A. The percentage of total axons measured was plotted against the axon core diameter distribution between 0.5 microns and 9.5 microns. The axon distributions at the proximal, middle, and distal sections were plotted for each animal.

4.1.3.1 8 weeks The fiber diameter histograms (FDH) were generated for two animals in group IV, three animals in group III, two animals in group II, and one group I normal control animal. In group IV, the distal distributions were grouped at larger diameters (5 microns) as compared to the middle and proximal distributions. In group III animals, the distributions at all the three sections were grouped between 2 and 5.5 microns, with maximum axon numbers occurring between 3 and 3.5 microns. In group II animals, the distributions were mostly grouped between 3 and 5 microns. Most of the diameters were distributed between 3.5 and 6.5 microns in the normal control.

4.1.3.2 12 weeks FDH were generated for two animals in group V, two animals in group III, two animals in group II, and one normal control animal in group I. In group V, the distal distributions were grouped at larger diameters (between 3 and 3.5 microns) in contrast to the middle and proximal distributions. The proximal distributions were grouped at the lower diameters (between 2 and 2.5 microns), with the middle distributions grouped between the proximal and distal distributions. In group III, the three distributions were grouped closely between 3 and 5 microns.
Similarly, in group II also, the distributions were grouped closely between 2.5 and 4.5 microns. In the normal control, the diameter spread was between 3.5 and 6.5 micron, with the maximum distribution occurring between 4 and 4.5 microns.

4.1.3.3 16 weeks FDH were generated for 4 animals in group VI, 4 animals in group III, 2 animals in group II, and one animal in group I. In group VI, animal #12 showed a bimodal distribution of the proximal axons, with the peaks occurring at 1.5 and 3.0 microns. The proximal distributions in the other three animals exhibited mostly the bell-shaped, Gaussian distribution. Similarly, in the same group, animal #14 showed a bimodal distribution of the distal axons with peaks located at 2.0 and 4.0 microns. In group III, the distributions at the three sections showed the bell-shaped distributions, with most of the grouping occurring between 1.5 and 5.0 microns. The distal distributions were located at larger diameters (between 4 and 5 microns), as compared to the middle and proximal distributions. Group II also exhibited the bell-shaped distributions at all the three sections with most distributions occurring between 1.5 and 5.5 microns. In group I, the normal control distribution was spread between 2.5 and 7.0 microns with the peak occurring at 5 microns.

4.1.3.4 24 weeks FDH were generated for 3 animals in group VII, 4 animals in group III, 2 animals in group II, and one animal in group I. In group VII, the distal distributions occurred at larger diameters (between 2 and 5.5 microns), the middle distributions were located between 2 and 4.5 microns, and the proximal distributions were located at the smaller diameters (between 1.5 and 4 microns). In group III, the distributions from the three sections were grouped in a bell-shaped distribution between 1.5 and 5.5 microns. In group II, the bell-shaped distributions were grouped
between 1 and 9 microns in one animal, indicating a wide spread, and between 2 and 6.5 microns in another animal. The normal control was distributed between 1.5 and 9 microns with peaks located at 3.5 and 5.5 microns.

The histograms showing mean axon counts, mean cross-sectional areas of axons, mean axon core diameters, and mean cross-sectional area of the nerve for the four time periods and for the repair methods are displayed in Appendix B. The corresponding data for the four normal controls are also shown.

4.1.4 Quantitative results

The results from the automated image analysis are listed in Table 4.2, Table 4.3 and Table 4.4. Table 4.2 lists the axonal data at the three sections, such as the total number of axons counted in the entire cross-section, the mean axonal core diameters of those axons, and the total cross-sectional area of all the axons that were counted. Table 4.3 lists the number of tissue strands inside the multiple-lumen cuffs and the total cross-sectional area of the entire regenerated nerve at the proximal, middle, and distal sections. Table 4.4 lists the diameter and mean cross-sectional area of the tissue strands retrieved from the multiple-lumen cuff repairs. Note that in the case of group I animals (i.e., normal controls), the quantitative data were obtained from tissue sections taken from only one site in the right sciatic nerve which was comparable to the middle of the repair site in the other groups. The results are grouped according to implantation periods in all the tables. Means and standard deviations (SD) are also listed for the data from the three repair methods.
Table 4.2: Number, mean diameter, and cross sectional area of axons for all groups

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<th>Implant Period &amp; Type of Repair</th>
<th>Animal Number</th>
<th>Axon Counts</th>
<th>Mean Axon Diameter (μm)</th>
<th>Total Cross Sectional Area of Axons (μm²)</th>
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<td>Prox  Mid  Dist</td>
<td>Prox  Mid  Dist</td>
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<td></td>
<td></td>
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ML=Multiple-lumen cuff; SL=Single-lumen cuff; EE=End-to-end repair; NC=Normal control.

bNot Available.
cSD=Standard Deviation.
dNot Applicable.
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<th>Total Cross Sectional Area of Axons (μm²)</th>
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Table 4.2 (Continued)
Table 4.2 (Continued)

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Table 4.2 (Continued)
Table 4.3: Total cross sectional areas of the regenerated nerve at the proximal, middle, and distal sections

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</tr>
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</tr>
<tr>
<td>Lumen</td>
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<td>-</td>
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</tr>
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</tr>
<tr>
<td>End to End</td>
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<td>37</td>
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<tr>
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<td></td>
<td></td>
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</tr>
<tr>
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<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>0.70±0.5</td>
</tr>
<tr>
<td>Single</td>
<td></td>
<td>25</td>
<td>-</td>
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</tr>
<tr>
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<td>26</td>
<td>-</td>
<td>0.48</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43</td>
<td>-</td>
<td>0.83</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>44</td>
<td>-</td>
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<td>N/A</td>
</tr>
<tr>
<td>Mean ±SD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.66±0.2</td>
</tr>
<tr>
<td>End to End</td>
<td></td>
<td>27</td>
<td>-</td>
<td>0.59</td>
<td>1.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28</td>
<td>-</td>
<td>1.44</td>
<td>0.55</td>
</tr>
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<td>Mean ±SD</td>
<td></td>
<td></td>
<td></td>
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<td>1.01±0.4</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td>29</td>
<td>-</td>
<td>—</td>
<td>0.76</td>
</tr>
</tbody>
</table>

a Applies to multiple-lumen cuffs only.
b Not available.
c SD=Standard Deviation.
Table 4.3 (Continued)

<table>
<thead>
<tr>
<th>Implant Period &amp; Type Of Repair</th>
<th>Animal Number</th>
<th>Number Of Strands Inside Cuff</th>
<th>Total Cross Sectional Area of Nerve (mm²)</th>
<th>Proximal</th>
<th>Middle</th>
<th>Distal</th>
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</thead>
<tbody>
<tr>
<td>16 Weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple</td>
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<td>0.2492</td>
<td>0.86</td>
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<tr>
<td>Lumen</td>
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<td>0.1828</td>
<td>0.18</td>
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</tr>
<tr>
<td></td>
<td>13</td>
<td>5</td>
<td>1.03</td>
<td>0.1814</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>7</td>
<td>1.11</td>
<td>0.2247</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Mean ±SD</td>
<td></td>
<td></td>
<td>1.23±0.4</td>
<td>0.22±0.1</td>
<td>0.42±0.3</td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>15</td>
<td>-</td>
<td>0.23</td>
<td>0.40</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>Lumen</td>
<td>16</td>
<td>-</td>
<td>0.34</td>
<td>0.36</td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>-</td>
<td>0.36</td>
<td>0.53</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>-</td>
<td>0.93</td>
<td>0.21</td>
<td>1.10</td>
<td></td>
</tr>
<tr>
<td>Mean ±SD</td>
<td></td>
<td></td>
<td>0.47±0.3</td>
<td>0.37±0.1</td>
<td>0.90±0.2</td>
<td></td>
</tr>
<tr>
<td>End to End</td>
<td>17</td>
<td>-</td>
<td>0.55</td>
<td>1.02</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>-</td>
<td>0.49</td>
<td>1.40</td>
<td>0.29</td>
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<tr>
<td>Mean ±SD</td>
<td></td>
<td></td>
<td>0.52±0.1</td>
<td>1.21±0.2</td>
<td>0.52±0.2</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>19</td>
<td>-</td>
<td>-</td>
<td>0.82</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>24 Weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>1</td>
<td>2</td>
<td>1.11</td>
<td>0.0505</td>
<td>0.57</td>
<td></td>
</tr>
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<td>6</td>
<td>1.71</td>
<td>0.1616</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
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<td>3</td>
<td>5</td>
<td>1.07</td>
<td>0.1345</td>
<td>0.59</td>
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</tr>
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<td>0</td>
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<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Mean ±SD</td>
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<td></td>
<td>1.30±0.3</td>
<td>0.09±0.1</td>
<td>0.55±0.1</td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>5</td>
<td>-</td>
<td>0.97</td>
<td>0.37</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>Lumen</td>
<td>6</td>
<td>-</td>
<td>0.52</td>
<td>0.29</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>-</td>
<td>0.73</td>
<td>0.45</td>
<td>0.61</td>
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</tr>
<tr>
<td></td>
<td>48</td>
<td>-</td>
<td>0.74</td>
<td>0.41</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>Mean ±SD</td>
<td></td>
<td></td>
<td>0.74±0.2</td>
<td>0.38±0.1</td>
<td>0.65±0.2</td>
<td></td>
</tr>
<tr>
<td>End to End</td>
<td>7</td>
<td>-</td>
<td>0.68</td>
<td>0.77</td>
<td>1.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>-</td>
<td>0.81</td>
<td>1.28</td>
<td>1.32</td>
<td></td>
</tr>
<tr>
<td>Mean ±SD</td>
<td></td>
<td></td>
<td>0.74±0.1</td>
<td>1.02±0.3</td>
<td>1.25±0.1</td>
<td></td>
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<tr>
<td>Normal</td>
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<td>-</td>
<td>-</td>
<td>1.13</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.4: Diameter and mean cross sectional area of tissue strands retrieved from the multiple-lumen cuffs for all groups

<table>
<thead>
<tr>
<th>Implant Period</th>
<th>Animal Number</th>
<th>Number Of Strands</th>
<th>Strand Diameter (mm)</th>
<th>Mean ± SD&lt;sup&gt;a&lt;/sup&gt; Strand Cross Sectional Area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 Weeks</td>
<td>31</td>
<td>1</td>
<td>0.147</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>1</td>
<td>0.131</td>
<td>0.01</td>
</tr>
<tr>
<td>12 Weeks</td>
<td>22</td>
<td>3</td>
<td>0.166 0.188 0.143</td>
<td>0.166 ± 0.02 0.022 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>7</td>
<td>0.259 0.176 0.222</td>
<td>0.219 ± 0.04 0.039 ± 0.01</td>
</tr>
<tr>
<td>16 Weeks</td>
<td>11</td>
<td>6</td>
<td>0.241 0.249 0.217</td>
<td>0.229 ± 0.02 0.042 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>5</td>
<td>0.203 0.192 0.232</td>
<td>0.215 ± 0.02 0.037 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>5</td>
<td>0.156 0.215 0.244</td>
<td>0.213 ± 0.03 0.036 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>7</td>
<td>0.217 0.217 0.185</td>
<td>0.201 ± 0.02 0.032 ± 0.01</td>
</tr>
<tr>
<td>24 Weeks</td>
<td>1</td>
<td>2</td>
<td>0.203 0.153</td>
<td>0.178 ± 0.03 0.025 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6</td>
<td>0.213 0.161 0.214</td>
<td>0.180 ± 0.05 0.027 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>0.148 0.168 0.111</td>
<td>0.171 ± 0.07 0.027 ± 0.03</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean cross sectional area of all the tissue strands inside the multiple-lumen cuff.

<sup>b</sup>SD=Standard Deviation.

<sup>c</sup>Not Applicable.
The analysis of variance test (ANOVA) and the t-test (at the 0.05 level) showed a significant increase in the number of axons in the proximal ($p = 0.0461$, $F$ distribution value $= 4.52$, degrees of freedom $= 1$), middle ($p = 0.0043$, $F$ distribution value $= 10.38$, degrees of freedom $= 1$), and distal ($p = 0.0008$, $F$ distribution value $= 15.61$, degrees of freedom $= 1$) sections for all repair methods over the four time periods, with the increase being the greatest for axon counts in the middle section. No significant difference was seen among the repair methods for axon counts in the proximal and distal sections. The tests showed a significant difference in repair methods for axon counts at the middle section ($p = 0.0344$, $F$ distribution value $= 4.01$, degrees of freedom $= 2$), with the single-lumen repair method showing the highest counts followed by the multiple-lumen cuff repair. The end-to-end repair showed the lowest axon counts in the middle sections. No significant increase in the mean axon core diameters were seen at the three sections for the repair methods over the four time periods. However, a significant difference was seen among the repair methods for the mean axon core diameter at the proximal ($p = 0.0028$, $F$ distribution value $= 6.62$, degrees of freedom $= 3$) and middle ($p = 0.0127$, $F$ distribution value $= 5.47$, degrees of freedom $= 2$) sections. The t-tests (at the 0.05 level) showed that, at the proximal section, all three repairs had significantly lower mean axon core diameters compared to the normal controls, and the end-to-end repairs had significantly higher mean axon core diameters than the multiple-lumen repairs. Among the three repairs, at the proximal section, the end-to-end repair had the highest mean axon core diameter, followed by the single-lumen repair, and the multiple-lumen repair, respectively. At the middle section, the end-to-end repair again had a significantly higher mean axon core diameter, followed by the multiple-lumen and single-lumen
repairs, respectively. No significant differences among repairs were seen at the distal section for the mean axon core diameters. Also, the differences in axon counts at the proximal and distal, proximal and middle, and middle and distal sections were tested statistically to determine the effects of repair and time periods on axonal branching. No significant differences were noted for the different repairs and time periods.

The ANOVA and t-tests (at the 0.05 level) were also done on the axonal areas and nerve cross-sectional areas. The tests showed a significant increase in the axonal cross-sectional areas in the proximal ($p = 0.0001$, $F$ distribution value $= 25.17$, degrees of freedom $= 1$), middle ($p = 0.0140$, $F$ distribution value $= 7.25$, degrees of freedom $= 1$), and distal ($p = 0.0052$, $F$ distribution value $= 9.82$, degrees of freedom $= 1$) sections for all repair methods over the four time periods. The increase was the greatest for axonal areas in the proximal and middle sections over times. However, the tests showed a significantly higher axonal cross-sectional area for normal controls compared to the three repair methods only at the proximal section ($p = 0.0004$, $F$ distribution value $= 9.70$, degrees of freedom $= 3$). No significant differences were seen at the three sections among the three repair methods over time. No significant increase in total nerve cross-sectional area was seen in the repair methods over time. However, a significant difference in nerve cross-sectional area was noted among repair methods at the proximal ($p = 0.0461$, $F$ distribution value $= 3.19$, degrees of freedom $= 3$) and middle ($p = 0.0001$, $F$ distribution value $= 30.60$, degrees of freedom $= 2$) sections. At the proximal section, the nerve cross-sectional area was highest for the multiple-lumen repairs, followed by single-lumen repairs, and end-to-end repairs, respectively. On the other hand, at the middle section, the reverse was true, with the end-to-end repairs having the highest nerve cross-sectional areas followed by
the single-lumen and multiple-lumen repairs, respectively. Likewise, although not significantly different, the distal nerve cross-sectional areas were highest in the end-to-end repairs, followed by the single-lumen and multiple-lumen repairs, respectively.

The diameters of the tissue strands obtained from the middle of the multiple-lumen cuff repairs varied from 0.11 mm to 0.31 mm. The mean diameters were the largest in the 16 weeks post-implantation period animals in comparison to the other time periods. None of the diameters were large enough to fill the entire lumen diameter of 0.381 mm. Even though the mean strand diameters increased progressively from 8 weeks to 16 weeks, the mean diameters in the 24 weeks animals were smaller than that of the 16 weeks animals.

4.1.5 Electrophysiological results

The results from the quantitative electrophysiological evaluations are listed in Table 4.1. As mentioned under Materials and Methods, the ICAP percentages gave a comparison measure of functional regeneration, it does not, however, provide an absolute anatomical percentage of axons with distal connections, nor does it measure the axonal regeneration to end-organs. The area ratios for the four normal controls had a mean of 93.3 % (SD = 22.7), and a range between 58 % and 114.6 %. The ANOVA test done on the ICAP percentages did not show significant differences for the repair methods and the four time periods. This was because of the large range (between 26 % and 140 %) over which the ratios were distributed. No significant increase in ICAP percentages was seen in the repair methods over time.

The conduction velocity data was not tested statistically because not enough measurements were available for testing. See Table 4.1 for a listing of the conduction
velocities. The conduction velocity measured proximally in the four normal controls had a mean of 65.6 m/s (SD = 41.8) and a range between 33.3 m/s and 137.5 m/s. Distally, the conduction velocity measured in the normal controls had a mean of 38.8 m/s (SD = 11.1) and ranged between 30 m/s and 57.5 m/s. The proximal conduction velocity data for the animals that underwent surgery ranged between 15.4 m/s and 137.5 m/s, and the distal data varied from 25 m/s to 66.7 m/s. In several of the animals, the distal conduction velocity was not measured because of the limitations of the electrophysiological experimental set-up. Proximally, even though the mean conduction velocity for the repair methods increased over time from 12 to 24 weeks, the mean distal conduction velocity did not increase over the same period of time.

4.1.6 Videotape results

The rankings assigned by the five observers for the animals that were videotaped are listed in Table 4.5. The average of all five ranks assigned to each animal in the 16 and 24 weeks post-implantation periods was used in the ANOVA and t-tests (at the 0.05 level). No significant difference was seen in the ranking over the 12 and 24 weeks post-implantation time periods tested. However, a significant difference in ranking was noted among the three repair methods ($p = 0.0201$, $F$ distribution value = 5.51, degrees of freedom = 2). The mean ranking of the multiple-lumen repairs (mean = 1.7) was the nearest to 3, which was the rank assigned to normal controls, followed by the single-lumen repairs (mean = 0.9) and the end-to-end repairs (mean = 0.9).

The deviation of the rankings assigned to the animals from the value 3 (which was the rank assigned to normal controls) was also calculated. The ANOVA and t-tests (at the 0.05 level) were done on these deviation values. Similar to the ranking
procedure, the average of all the five deviation values was used in these tests. A significant difference ($p = 0.0201$, $F$ distribution value = 5.51, degrees of freedom = 2) in the deviation values was seen among the three repair methods. Similar to the ranking results, the mean deviation value of the multiple-lumen repairs (mean = 1.3) was the smallest, indicating that it was the closest to the normal control value of 3, followed by the single-lumen repairs (mean = 2.1) and the end-to-end repairs (mean = 2.1).

4.2 Discussion

The results of this study show that axons regenerated across a 5 mm gap inside a multiple-lumen silicone rubber nerve cuff in the adult rat sciatic nerve. Although the outcome varied significantly in the various evaluation methods used, it was clear that all of these evaluation methods showed evidence of axonal regeneration through the multiple-lumen cuffs at all four time periods studied. The histology evaluations confirmed the presence of axons across the repair sites in the multiple-lumen cuff repairs, the electrophysiological studies indicated some restoration of conduction properties, and the videotape evaluation was an indicator of functional recovery in the limb.

In the present experimental multiple-lumen cuff model, the regenerating nerve fibers formed several nerve bundles inside the cuff. The bundles showed a two zone reorganization, with an outer peripheral zone constituted mainly by connective tissue and numerous blood vessels, and an inner central zone which contained the neural elements.
Table 4.5: Videotape recording ranks assigned by the observers and their comments

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Type* of Repair</th>
<th>Implant Period (Weeks)</th>
<th>Rankb Assigned By Each Observer</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>41</td>
<td>SL</td>
<td>8</td>
<td>0 0.5 0 0.5 0.5</td>
<td>Curled digits, avoids weight on leg.</td>
</tr>
<tr>
<td>42</td>
<td>SL</td>
<td>8</td>
<td>1 0.5 0 1 1</td>
<td>Curled digits, spastic, exaggerated gait.</td>
</tr>
<tr>
<td>43</td>
<td>SL</td>
<td>12</td>
<td>2 1 2 2 2.5</td>
<td>Curled digits, puts weight on leg, gait almost normal.</td>
</tr>
<tr>
<td>11</td>
<td>ML</td>
<td>16</td>
<td>3 1 2 1 3</td>
<td>Curled digits, slightly exaggerated gait.</td>
</tr>
<tr>
<td>12</td>
<td>ML</td>
<td>16</td>
<td>1 0.5 1 1 2</td>
<td>Curled digits, puts weight on leg, hopping gait.</td>
</tr>
<tr>
<td>13</td>
<td>ML</td>
<td>16</td>
<td>3 2 2 1 3</td>
<td>Digits less curled, slightly exaggerated gait.</td>
</tr>
<tr>
<td>14</td>
<td>ML</td>
<td>16</td>
<td>1 2 1 1 2</td>
<td>Digits less curled, avoids weight on leg, hopping gait.</td>
</tr>
<tr>
<td>15</td>
<td>SL</td>
<td>16</td>
<td>0 0 1 0.5 0.5</td>
<td>Severely curled digits, avoids weight on leg, spastic, hopping gait.</td>
</tr>
<tr>
<td>16</td>
<td>SL</td>
<td>16</td>
<td>1 0.5 0 0.5 1</td>
<td>Curled digits, leg movements not controlled, hopping gait.</td>
</tr>
<tr>
<td>45</td>
<td>SL</td>
<td>16</td>
<td>1 0.5 1 2 1</td>
<td>Curled digits, avoids weight on leg, spastic, leg movements not controlled.</td>
</tr>
<tr>
<td>46</td>
<td>SL</td>
<td>16</td>
<td>3 2 1 1 2</td>
<td>Curled digits, leg movements slowed.</td>
</tr>
<tr>
<td>17</td>
<td>EE</td>
<td>16</td>
<td>3 1 1 1 3</td>
<td>Curled digits, avoids weight on leg, slowed leg movements.</td>
</tr>
<tr>
<td>18</td>
<td>EE</td>
<td>16</td>
<td>1 0.5 0 1 2</td>
<td>Curled digits, puts little weight on leg, slow and uncontrolled leg movements.</td>
</tr>
</tbody>
</table>

*ML=Multiple-lumen cuff; SL=Single-lumen cuff; EE=End-to-end repair; NC=Normal control.

b0=Worst case; 1=Decreased strength; 2=Some gait abnormalities; 3=Normal.
Table 4.5 (Continued)

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Type Of Repair</th>
<th>Implant Period (Weeks)</th>
<th>Rank Assigned By Each Observer</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>NC</td>
<td>16</td>
<td>3 3 3 3 3</td>
<td>Normal gait.</td>
</tr>
<tr>
<td>1</td>
<td>ML</td>
<td>24</td>
<td>3 2 1 2 2.5</td>
<td>Digits less curled, spastic, hopping gait.</td>
</tr>
<tr>
<td>2</td>
<td>ML</td>
<td>24</td>
<td>2.5 1 1 2 2.5</td>
<td>Digits less curled, spastic, avoids weight on leg, leg movements slowed.</td>
</tr>
<tr>
<td>3</td>
<td>ML</td>
<td>24</td>
<td>2 1 2 2 2.5</td>
<td>Digits extended, slightly spastic, gait exaggerated.</td>
</tr>
<tr>
<td>4</td>
<td>ML</td>
<td>24</td>
<td>1 0.5 1 1 2</td>
<td>Curled digits, spastic, hopping gait.</td>
</tr>
<tr>
<td>5</td>
<td>SL</td>
<td>24</td>
<td>1 0.5 1 1 1</td>
<td>Curled digits, leg movements not controlled, slightly exaggerated gait.</td>
</tr>
<tr>
<td>6</td>
<td>SL</td>
<td>24</td>
<td>0 1 0.5 0.5 0.5 1</td>
<td>Curled digits, puts little weight on leg, hopping gait.</td>
</tr>
<tr>
<td>7</td>
<td>EE</td>
<td>24</td>
<td>0 0 0 0.5 0.5</td>
<td>Curled digits, avoids weight on leg, spastic, drags leg while walking.</td>
</tr>
<tr>
<td>8</td>
<td>EE</td>
<td>24</td>
<td>0 0.5 0 1 2</td>
<td>Curled digits, avoids weight on leg, leg movements not controlled, hopping gait.</td>
</tr>
<tr>
<td>9</td>
<td>NC</td>
<td>24</td>
<td>3 3 3 3 3</td>
<td>Normal gait.</td>
</tr>
</tbody>
</table>
This kind of organization was also seen in the single-lumen controls and was similar to that reported by Jenq and Coggeshall (1985, 1987); Williams et al. (1983, 1984); Seckel et al. (1984); Madison et al. (1988); and Lundborg et al. (1982a). A marked difference between the experimental model and the single-lumen and end-to-end repair controls was evident in the distal stump's reorganization. Whereas, in the controls, the nerve regenerated through the distal stump forming one central, large, nerve bundle (or fascicle), in the experimental model, the axons regenerated through the distal stump forming several such smaller nerve bundles whose orientation was determined by the pattern of holes in the multiple-lumen cuff. Thus, the multiple-lumen model offered the advantage of predisposing the orientation of the regenerating nerve before it entered the distal stump. Also, the crossing over of the regenerating axons and neuroma formation at the repair site that were evident in the end-to-end repairs was absent in the multiple-lumen cuff model and the single-lumen cuff controls.

The fiber diameter histograms indicated a preponderance of the larger diameter (between 3 and 5 microns) axons in the multiple-lumen cuff models, especially at the 12 and 24 weeks post-implantation periods, at the middle and distal sections. This could be because the regenerating axons matured faster in the experimental model, or the multiple-lumen cuff repair favored the regeneration of the larger α motor neurons and Group-I sensory neurons over the smaller sensory neurons. Also, the bimodal distributions seen in the experimental models at the 16 weeks implant period suggest the simultaneous regeneration of two distinct fiber sizes. This kind of bimodal distribution was also reported by Fields and Ellisman (1986b) after 12 weeks. In their research, by 40 weeks, the FDH of their experimentally repaired nerves was similar.
to that of the normal nerve, indicating that all major fiber groups had undergone regeneration by that time, and had attained nearly normal relative sizes. However, the absolute size of axons in all fiber groups was less than in normal nerves. Although the spread of the axon distributions did not significantly increase over time, in our study, in the 16 and 24 weeks time periods, there were indications of distributions occurring on either side of the grouping seen in the earlier 8 and 12 weeks time periods.

The quantitative results showed a significant increase in regenerated axon populations over the four time periods studied. This correlates with significant increases in axonal cross-sectional areas measured over the four time periods, for all repair groups, at all the three regions evaluated. Although the regenerated axon populations were significantly higher at the middle section for the single-lumen repairs, this did not manifest into a larger nerve cross-sectional area at the middle section, indicating greater axon densities in the regenerated nerve. The mean core diameter of the regenerated axon populations in the repaired nerves did not reach the normal control levels even after 24 weeks. This is not surprising, because other researchers like Fields and Ellisman (1986b), Henry et al. (1985), Le Beau et al. (1988b), and Rosen et al. (1983, 1989) have observed the same results for even longer implantation periods, and some of them have concluded that the regenerated axon populations never reach the normal levels. The higher mean regenerated axonal core diameters, at the proximal and middle sections, seen in the end-to-end repairs suggests a faster maturation rate for this type of repair. Also, since the regenerating axons in the end-to-end repairs did not initially have to cross a 5 mm gap, and since the distal stump had a greater trophic influence on the regenerating axons because of its closer
proximity in this case, the maturation rates could have been faster for end-to-end repairs as compared to the cuff repairs. The statistical tests on the differences in axon counts at the proximal, middle, and distal sections did not show evidence of either excessive branching or the inability of a large number of proximal axons to regenerate, for all the repair methods. If either of these cases were true, it would have manifested as higher or lower axonal numbers at the middle and distal sections, respectively.

The larger nerve cross-sectional area seen proximally, in the multiple-lumen cuff models, may be due to the way in which the axons regenerate across the gap. In the multiple-lumen cuff model, instead of one central nerve bundle, the regenerating axons group into several circularly dispersed nerve bundles. This pattern of regeneration may be responsible for the wider proximal nerve cross-sections in the experimental model. The larger middle nerve cross-sectional area in the end-to-end repairs can be attributed to the neuroma formation at the repair site. The multiple-lumen models had the smallest nerve cross-sectional area, both in the middle and distal regions. This is in concurrence with the 2.5x reduction in total cross-sectional area of the multiple-lumen tubes as compared to the single-lumen tubes. However, a comparable decline in regenerated axonal populations was not seen in the multiple-lumen experimental models as compared to the single-lumen controls. Thus, there are comparable axonal counts at the middle sections for the experimental models, in spite of the reduced nerve cross-sectional areas. This indicates that the axonal density of the multiple-lumen cuff repairs are greater than that seen in either single-lumen or end-to-end repairs. The mean strand diameters of the tissue strands increased over time although the mean diameters of the 24 weeks animals were smaller than those
of the 16 weeks animals. However, the 24 weeks implant group had the largest strand diameter of 0.306 mm, and it is possible that the strand diameters increase in size over time as more and more axons mature and remyelination occurs.

The electrophysiological results were not conclusive, but they indicated restoration of conduction properties, and a degree of functional recovery across the regenerated nerve. The ICAP ratios obtained from normal controls (mean = 93.3±22.7) were comparable to those observed by Ashur et al. (1987), and Rosen et al. (1979). The large deviations observed in this study can be attributed to several sources of error due to technical difficulties in recording weak electrical responses of fragile regenerating axons and because of the numerous assumptions regarding the current path through the nerve from compound action potential responses. For example, a larger portion of the applied stimulating current could have been shunted through the low-resistance extra-cellular path in regenerated nerves of different ages if axons are less dense than normal. The ICAP ratios obtained from the repaired nerves in this study were also comparable with the results reported by Ashur et al. (1987), Marshall et al. (1989), Rosen et al. (1979, 1989), and Sabelman et al. (1989).

The mean conduction velocities measured proximally (mean = 44 m/s) in the repaired nerves were lower than those measured proximally in normal controls (mean = 65.6 m/s). However, the mean conduction velocities measured distally (mean = 46 m/s) in the repaired nerves were greater than those measured distally in normal controls (mean = 38.8 m/s). There was no evidence, either in the mean axonal diameters (which may indicate axon maturation) or in the axon distributions (which may indicate larger diameter regenerated axon populations) to substantiate the discrepancy mentioned above between repaired and normal nerves. The conduction velocities
measured in the repaired nerves were comparable to the \textit{in vivo} results reported by Chiu et al. (1982, 1988), Cuadros and Granatir (1987), Fields and Ellisman (1986a), and Lundborg et al. (1982b).

The videotape evaluation, although relative, was a totally blind test and was an indicator of a degree of functional recovery in the leg after nerve transection and repair. Both the ranking and deviation from the normal tests favored the multiple-lumen cuff model over the single-lumen cuff and end-to-end repair controls at the 16 and 24 weeks time periods studied. This suggests that the degree of functional recovery was comparable, if not better, in the experimental model, as compared to controls for corresponding time periods. In this study, estimations of axonal regeneration to end-organs were not evaluated because of the short-term nature of the time periods evaluated.
5. CONCLUSIONS

This study represents the initial steps of an attempt to utilize a multiple-lumen silicone rubber cuff to bridge a 5 mm gap in the adult rat sciatic nerve. A seven-lumen, double-tube, silicone rubber cuff was designed, fabricated, and in vivo tested in the right sciatic nerve of adult rats for 8, 12, 16, and 24 weeks post-implantation periods. Silicone rubber single-lumen cuffs were also implanted in control animals. Another group of control animals underwent end-to-end repair anastomosis after nerve transection. The current work was successful in bridging the 5 mm gap in the sciatic nerve of the rat using the multiple-lumen cuff experimental models at all four post-implantation periods studied. Four evaluation methods, videotape rankings, electrophysiological tests, qualitative, and quantitative histology were used to evaluate and compare the results of the study. The electrophysiological studies indicated restoration of conduction properties in the regenerated axons across the repair site. However, the large variability and unreliability of the quantitative electrophysiological data made detailed comparisons impossible among the groups and hence meaningful conclusions with respect to ICAP ratios and conduction velocities cannot be drawn from this data. The videotape results from the 16 and 24 weeks post-implantation animals showed a degree of functional recovery in the affected limb after nerve transection and repair. The videotape ranking results favored the experimental model
over the controls. The qualitative and quantitative evaluations confirmed the regeneration of axons through the lumen of the experimental model. The qualitative histology results showed that the regenerating axons formed several nerve bundles as they regenerated across the gap in the experimental model. Each of these nerve bundles filled a lumen in the multiple-lumen cuff, and their reorientation was determined by the pattern of holes inside the cuff. Thus, the experimental multiple-lumen cuff helped in initially determining the pattern of regeneration of the axons across the repair site. The same patterns of regeneration seen at the repair site were carried through to the distal stump in the multiple-lumen cuff repairs. Axon counts were comparable to control levels at the repair sites in the experimental model in spite of a 2.5x reduction in the cross-sectional area available for regeneration across the gap. This indicated higher axonal densities in the cross-sections of the experimental model. Neither excessive branching nor loss of proximal axons in large numbers were evidenced in the experimental cuff model as the regenerating axons crossed the gap. Mean fiber core diameters did not reach normal control levels at the end of the four time periods tested. The experimental multiple-lumen cuff model in this study can be termed as successful because the multiple-lumen cuff was able to sustain axonal regeneration across a 5 mm gap in the rat sciatic nerve, with minimum to moderate host reaction, with no neuroma formation at the repair site, and with comparable histologic structures and axonal counts at the repair sites compared to those seen in the control single-lumen cuff repairs.

Future research on this project might include using different patterns of holes in the multiple-lumen cuff to study interaction between hole layout and reorganization of the regenerating axons, substituting the saline in the lumen of the cuffs with
a medium containing neuronotrophic factors that enhance nerve regeneration, and conducting long term studies (12 to 24 months) to evaluate axonal regeneration to end-organs. Also, a bio-resorbable multiple-lumen cuff could be fabricated to initially reorient the regenerating axons and then as it disappears, provide more cross-sectional area for the maturation of axons. Finally, the multiple-lumen cuffs could be used to simultaneously study the interaction between regenerating axons and other neural and non-neural factors that influence regeneration, by providing a different but controlled environment for regeneration, in each of the lumens of the nerve cuff.
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ACKNOWLEDGEMENTS

I am greatly indebted to my major professor, Dr. Raymond T. Greer, for giving me the opportunity to conduct this research work under his guidance. During my studies at Iowa State University, he has been an endless source of knowledge, encouragement and support. I would like to thank Drs. Mary Helen Greer, Robert Carithers, Frederick Hembrough and Etsuro Uemura for serving on my committee and for all their valuable suggestions and assistance. I would also like to thank Dr. Charles Drewes and Dr. Philip Pearson for their expert advice and Dr. Raymond Kudej for performing the animal surgeries. I also extend my sincere thanks to the Biomedical Engineering Program for supporting this work and to all the faculty and students for their advice and assistance.

Finally, I would most like to thank my family, especially my mother, for the tremendous support and unending encouragement they gave me throughout my studies. I would like to express my love, admiration and gratitude to them for making it all worth it. They have been a great part of my efforts to get where I am today.

This research is dedicated to my mother for her endless love, endurance, and dedication, and for inspiring all her children to strive for excellence in their careers.
APPENDIX A: FIBER DIAMETER HISTOGRAMS
Axon Diameter Distribution
Animal #31, Group (IV)
8 Weeks, Multiple-Lumen Cuff

Axon Diameter Distribution
Animal #34, Group (IV)
8 Weeks, Multiple-Lumen Cuff
Axon Diameter Distribution
Animal #36, Group (III)
8 Weeks, Single-Lumen Cuff

Axon Diameter Distribution
Animal #41, Group (III)
8 Weeks, Single-Lumen Cuff
Axon Diameter Distribution
Animal #42, Group (III)
8 Weeks, Single-Lumen Cuff
Axon Diameter Distribution
Animal #37, Group (II)
8 Weeks, End-To-End Repair

Axon Diameter Distribution
Animal #38, Group (II)
8 Weeks, End-To-End Repair
Axon Diameter Distribution
Animal #39, Group (I)
8 Weeks, Normal Control

Percentage Axons

Axon Core Diameter (Microns)
Axon Diameter Distribution
Animal #22, Group (V)
12 Weeks, Multiple-Lumen Cuff

Axon Diameter Distribution
Animal #24, Group (V)
12 Weeks, Multiple-Lumen Cuff
Axon Diameter Distribution
Animal #26, Group (III)
12 Weeks, Single-Lumen Cuff

Axon Core Diameter (Microns)

Percentage Axons

Axon Diameter Distribution
Animal #43, Group (III)
12 Weeks, Single-Lumen Cuff

Axon Core Diameter (Microns)
Axon Diameter Distribution
Animal #27, Group (II)
12 Weeks, End-To-End Repair

Axon Diameter Distribution
Animal #28, Group (II)
12 Weeks, End-To-End Repair
Axon Diameter Distribution
Animal #29, Group (I)
12 Weeks, Normal Control

Percentage Axons

Axon Core Diameter (Microns)
Axon Diameter Distribution
Animal #11 (Group VI)
16 Weeks, Multiple-Lumen Cuff

Axon Diameter Distribution
Animal #12 (Group VI)
16 Weeks, Multiple-Lumen Cuff
Axon Diameter Distribution
Animal #13 (Group VI)
16 Weeks, Multiple-Lumen Cuff

Axon Diameter Distribution
Animal #14 (Group VI)
16 Weeks, Multiple-Lumen Cuff
Axon Diameter Distribution
Animal #15 (Group III)
16 Weeks, Single-Lumen Cuff

Axon Diameter Distribution
Animal #16 (Group III)
16 Weeks, Single-Lumen Cuff
Axon Diameter Distribution
Animal #45 (Group III)
16 Weeks, Single-Lumen Cuff

Axon Diameter Distribution
Animal #46 (Group III)
16 Weeks, Single-Lumen Cuff
Axon Diameter Distribution
Animal #17 (Group II)
16 Weeks, End-To-End Repair

Percentage Axons

Axon Core Diameter (Microns)

Axon Diameter Distribution
Animal #18 (Group II)
16 Weeks, End-To-End Repair

Percentage Axons

Axon Core Diameter (Microns)
Axon Diameter Distribution
Animal #19 (Group I)
16 Weeks, Normal Control
Axon Diameter Distribution
Animal #1 (Group VII)
24 Weeks, Multiple-Lumen Cuff

Axon Diameter Distribution
Animal #2 (Group VII)
24 Weeks, Multiple-Lumen Cuff
Axon Diameter Distribution
Animal #3 (Group VII)
24 Weeks, Multiple-Lumen Cuff

Percentage Axons

Axon Core Diameter (Microns)
Axon Diameter Distribution
Animal #5 (Group III)
24 Weeks, Single-Lumen Cuff

Axon Diameter Distribution
Animal #6 (Group III)
24 Weeks, Single-Lumen Cuff
Axon Diameter Distribution
Animal #47 (Group III)
24 Weeks, Single-Lumen Cuff

Axon Diameter Distribution
Animal #48 (Group III)
24 Weeks, Single-Lumen Cuff
Axon Diameter Distribution
Animal #7 (Group II)
24 Weeks, End-To-End Repair

Axon Core Diameter (Microns) vs. Percentage Axons

Proximal, Middle, and Distal sections are represented.

Axon Diameter Distribution
Animal #8 (Group II)
24 Weeks, End-To-End Repair

Axon Core Diameter (Microns) vs. Percentage Axons

Proximal, Middle, and Distal sections are represented.
Axon Diameter Distribution
Animal #9 (Group I)
24 Weeks, Normal Control

Percentage Axons

Axon Core Diameter (Microns)
APPENDIX B: HISTOGRAMS OF MEANS
Mean Axon Counts
8, 12, 16, & 24 Weeks Implant Periods

Mean Axon Counts

ML(Prox) ML(Mid) ML(Dist) SL(Prox) SL(Mid) SL(Dist) EE(Prox) EE(Mid) EE(Dist) NC

ML=Multiple-Lumen, SL=Single-Lumen
EE=End-to-End Repair, NC=Normal Control
Mean Cross Sectional Area Of Axons
8, 12, 16, & 24 Weeks Implant Periods

Mean CS Area (square micrometer)

<table>
<thead>
<tr>
<th>Implant Periods</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 Weeks</td>
</tr>
<tr>
<td>12 Weeks</td>
</tr>
<tr>
<td>16 Weeks</td>
</tr>
<tr>
<td>24 Weeks</td>
</tr>
</tbody>
</table>

Type of Repair
ML=Multiple-Lumen, SL=Single-Lumen
EE=End-to-End Repair, NC=Normal Control
Mean Axon Diameter
8, 12, 16, & 24 Weeks Implant Periods

Mean Axon Diameter (microns)

ML-Multiple-Lumen, SL-Single-Lumen
EE-End-to-End Repair, NC-Normal Control
Mean Cross Sectional Area Of Nerve
8, 12, 16, & 24 Weeks Implant Periods

Mean CS Area (square mm)

- ML (Multiple-Lumen)
- SL (Single-Lumen)
- EE (End-to-End Repair)
- NC (Normal Control)

ML(Prox) ML(Mid) ML(Dist) SL(Prox) SL(Mid) SL(Dist) EE(Prox) EE(Mid) EE(Dist) NC

Implant Periods:
- 8 Weeks
- 12 Weeks
- 16 Weeks
- 24 Weeks

ML = Multiple-Lumen, SL = Single-Lumen
EE = End-to-End Repair, NC = Normal Control