TISSUE-ENGINEERED NERVE GRAFTS WITH A CAPILLARY-LIKE NETWORK

MARIE-NOËLLE HÉBERT-BLOUIN

Department of Neurology and Neurosurgery, Faculty of Medicine

Integrated Program in Neuroscience

McGill University, Montréal

December 2009

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science, Integrated Program in Neuroscience

ABSTRACT

Introduction

A nerve graft is occasionally necessary for the treatment of traumatic peripheral nerve lesions. The gold standard remains the autograft, despite the study of multiple nerve guides as alternatives. This thesis utilizes a tissue-engineering approach to design an autologous nerve guide, with or without a capillary-like network.

Material and Methods

Human fibroblasts and endothelial cells were used to produce a tubular structure with a capillary-like network. The potential for these different nerve guides produced with a tissue-engineering approach to support nerve regeneration was studied with the rat sciatic nerve model.

Results

Tubular structures with and without a capillary-like network, composed entirely of human cells, can be produced with a new tissue-engineering technique. Despite evidence of some axonal regeneration, preliminary in vivo studies in the rat were negative.

Conclusion

This thesis presents an innovative tissue-engineering technique for the production of autologous nerve guides from human cells, with a capillary-like network.

RESUME

Introduction

Une greffe nerveuse est parfois nécessaire pour le traitement des dommages aux nerfs périphériques. L'autogreffe demeure le traitement standard, malgré l'étude de plusieurs guides nerveux comme alternative. Cette thèse étudie la conception d'un guide tubulaire autologue, avec ou sans réseau de pseudo-capillaires, par une approche de génie tissulaire.

Matériel et méthodes

Des cellules humaines fibroblastiques et endothéliales ont été utilisées afin de produire une structure tubulaire avec un réseau de pseudo-capillaires. La régénération nerveuse supportée par les différents guides nerveux produits à partir du génie cellulaire a été testée avec le modèle de réparation nerveuse du nerf sciatique chez le rat.

Résultats

Des structures tubulaires avec un réseau de pseudo-capillaires, composées entièrement de cellules humaines, peuvent être produites en utilisant une nouvelle technique de génie tissulaire. Malgré l'évidence d'une certaine régénération axonale, les études préliminaires in vivo chez le rat sont négatives.

Conclusion

Cette thèse présente une approche de génie tissulaire innovatrice pour la création d'un guide nerveux autologue et possiblement endothélialisé à partir de cellules humaines de la peau.

ACKNOWLEDGEMENTS

I would like to thank first and foremost my supervisors and advisory committee, Dr. André Olivier, Dr. Line Jacques, and Dr. François Berthod for the wonderful opportunity to work on this project. I would also like to thank Dr. François Berthod and his laboratory, the LOEX (Laboratoire d'organogénèse expérimentale, Hôpital du Saint-Sacrement, Laval University), for sharing with me their knowledge, their techniques, their facilities and for the wonderful welcome, constant guidance, support, and advice throughout this project. I would like to thank Dr. René Caissie for sharing with me his advances and previous experiments that lead to the design of this thesis and his help with the surgical procedure, and Dr. Jacques for her guidance, her support and technical help with the surgical procedures. I also greatly appreciated the support of Dr. Olivier and of Dr. Jacques for starting this master as part of my neurosurgical residency program.

I also want to thank the team of the LOEX, and especially the Neuro team, for their friendship and advice. I especially thank Jean Dubé, who shared with me his knowledge and guided me throughout the design of these tubes, Rina Guignard, for teaching me all the techniques required for this thesis, Rosemarie LeMay-Tremblay, Florence Tomasetig, Vicky Gagnon, and Myriam Grenier, for their help throughout my thesis, Marie-France Champigny for keeping excellent records of all previous work done, and all the other members of the Neuro team for their all their advices. I would finally like to thank Anne-Marie Moisan for all her help with the in vivo study.

To my family, thank you for your moral support and encouragement. I am especially thankful to my mother, Louise Hébert, to my father, Michel Blouin, and to my husband, Mohamed Nosair, for their support and presence.

ABBREVIATIONS

BDNF: brain-derived neurotrophic factor

bFGF: basic fibroblast growth factor

DME: Dulbecco's modification of Eagle's medium

DMSO: dimethyl sulfoxide

GFP: green fluorescent protein

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HUVEC: Human umbilical vein endothelial cells

HUVEC-GFP: Human umbilical vein endothelial cells transfected with green fluorescent protein

ITS: intermediary toe spread

LOEX: Laboratoire d'organogénèse expérimentale

NGF: nerve growth factor

O.C.T.: optimal cutting temperature

PECAM-1: platelet endothelial cell adhesion molecule-1

PGA: polyglycolic acid

PL: print length

PLCL: poly-L-lactide-caprolactone

PLGA: poly-lactic-glycolic acid

PTFE: polytetrafluoroethylene

PVA: polyvinyl alcohol

RGMW: relative gastrocnemius muscle weight

TS: toe spread

LIST OF FIGURES AND TABLES

Figures

Chapter 3:

Chapter 4:

Tables

Chapter 2

Chapter 3

Chapter 4

TABLE OF CONTENT

CHAPTER 1 - INTRODUCTION

Thesis Rationale
Thesis Objective and Hypotheses
Thesis Organization

CHAPTER 1. INTRODUCTION

1.1 Thesis Rationale

Peripheral nerve lesions complicate 2 to 5% of traumatic lesions to the extremities[60,100]. As the nerves of the upper extremity (median, ulnar and radial nerves) are most commonly injured[100], these lesions lead to important and often catastrophic functional motor and sensory deficits. Despite advances in microsurgical techniques, the injured peripheral nerve remains a difficult tissue to repair. Several issues prevent the application of strategies used for the repair of other tissues: 1) the complexity of the nerve structure, 2) the rapidity of Wallerian degeneration and its consequences on the denervated muscle leading to atrophy which may become irreversible, 3) the susceptibility of nerves to ischemia, and 4) the susceptibility of nerves to mechanical factors such as traction and compression.

In the presence of an acute nerve laceration without retraction or loss of substance, the nerve stumps are approximated using epineurial or fascicular microsutures without an intervening nerve graft. However, in many clinical situations, a gap is present between nerve stumps, either due to retraction, loss of substance, or the necessity to remove a neuroma-in-continuity. In these cases, a nerve graft or nerve guide to bridge the nerve defect is necessary. Presently, the autologous nerve graft is the "gold standard"[66], because of its physiological architecture and viable Schwann cells. Various autologous donor nerves are available, the most common being the sural nerve[60]. However, autografts have several limitations including their limited availability, the associated donor site morbidity (scar, loss of sensation, painful neuroma, potential for neuropathic pain) and often their inappropriate diameter[60,119].

To palliate the limitations of the autografts, various alternatives have been developed, including the use of autogenous venous grafts[23], nerve allografts[85,118], and nerve tubes, guides or conduits. Despite multiple studies on the development of several synthetic nerve tubes, autografts remain the gold standard. Presently, the most promising nerve tubes are biodegradable nerve guide implants and five are approved for use in humans: *Neurotube* (polyglycolic acid), *NeuroMatrixNeuroflex* (collagen), *Neurolac* (poly-L-lactide-caprolactone), *Neurogen* (collagen), *Salubridge* (polyvynil alcohol

hydrogel)[112]. However, these nerve tubes are mainly applied in the repair of sensory nerves of small caliber and with short nerve gap distances (less than 3cm).

In this context, many in-vitro and animal experimental studies are in progress. Various materials are explored from extracellular matrix components to synthetic polymers, various models are studied including the use of multichannel or longitudinal filaments and the addition of recombinant proteins or cells producing growth factors are evaluated in an attempt to construct a nerve guide that could be an alternative to the autograft.

Another major issue important to address in the design of nerve guides of large caliber is how to ensure their vascularization. For nerves and nerve grafts of small caliber (<2mm), passive diffusion is sufficient for oxygen and nutrients supply as well as for toxin removal. However, larger nerves require a vascular supply as axons are very sensitive to hypoxia. This is a major obstacle to reinnervation through potentially large size nerve guides.

The need for the design of a nerve guide, which could support axonal regeneration, with an adequate vascularization, was the foundation for the design of this research project. The other foundation of this research project was the collaboration with the LOEX (Laboratoire d'organogénèse expérimentale, Hôpital du Saint-Sacrement, Laval University), which has a vast experience in tissue-engineering approaches. These tissue-engineering techniques, which were used to reconstruct blood vessels in-vitro[75], will be modified, as a simple tubular structure cannot support optimal nerve regeneration. Developing such an approach to produce nerve guides would have definite advantages. First, the possibility to use autologous cells has the advantage of eliminating all exogenous material that could lead to inflammatory and/or immune reactions or have a risk of viral transmission (like it is the case for bovine collagen). Furthermore, this strategy could lead to the development of a nerve guide composed of living cells. Nerve regeneration could be improved by the secretion of growth factor by the living cells (fibroblasts) from which they are constructed. Finally, the development of a technique to produce an internal capillary-like network within those nerve guides could significantly accelerate the speed of graft revascularization and therefore potentially improve axonal regeneration.

1.2 Thesis Objective and Hypotheses

This thesis objective is to evaluate the potential to develop a tubular biomaterial to support nerve regeneration in the context of peripheral nerve trauma using tissue-engineering approaches. These techniques were explored in the aim to produce reconstructed completely biological nerve guides from autologous or heterologous cells, with or without an internal capillary-like network. As this is a considerable task, this thesis is not meant to achieve this overall goal, but to start the design of these completely biological nerve guides

The specific hypotheses of this thesis are:

1) A tissue-engineering approach to produce reconstructed completely biological nerve guides from living autologous or heterologous cells can be developed.
2) An internal capillary-like network can be developed within these tissue-engineered nerve grafts.
3) The tissue-engineered nerve grafts could represent an alternative to autograft to support nerve regeneration.

1.3 Thesis Organization

This thesis organization follows, within each section, the three specific hypotheses described above. In chapter 2, a background discussion based on a comprehensive literature review on the anatomy of peripheral nerve, the pathophysiology of nerve injury, the physiology of nerve regeneration, the surgical strategies for nerve repair, and the different attempts at nerve tubulization is provided. The material and methods used to address each specific hypothesis are presented in chapter 3. Chapter 3 also includes a brief review of the previous achievements of the LOEX in the domain of bio-engineering relevant to the techniques used in this thesis. The main results obtained for each hypothesis are exposed in chapter 4. A general discussion of the thesis as well as possible future extensions of this research are presented in chapter 5. Finally, an overall conclusion is presented in chapter 6.

CHAPTER 2 – COMPREHENSIVE LITERATURE REVIEW

CHAPTER 2. COMPREHENSIVE REVIEW OF THE LITERATURE

This thesis focuses on the development of a tubular biomaterial to support nerve regeneration in the context of peripheral nerve trauma. In order to develop a tubular structure able to sustain nerve regeneration, a thorough understanding of the anatomy of peripheral nerve, the pathophysiology of nerve injury, the physiology of nerve regeneration, and the surgical strategies for nerve repair is necessary. Selected and basic considerations relevant to these elements will be briefly summarized here in this comprehensive literature review. In this thesis, an attempt to develop an internal capillary-like network that may accelerate the speed of graft revascularization is made. In this context, the normal neural blood supply will be discussed in the anatomy section. Moreover, the different significant attempts at nerve tubulization for repair of injuries with nerve gaps will be reviewed.

2.1 Anatomy of Peripheral Nerves

Peripheral nerves are pathways to and from the central nervous system. They constitute the efferent pathways for the motor and autonomic system and the afferent pathways for perception of position, pressure, touch, temperature, and pain[71]. Peripheral nerves are composed of nerve fibers and supporting tissue. Even if the nerve fibers are intuitively thought to be of more importance, both components are essential. In fact, the majority of the nerve volume is composed of connective tissue[71], which has an important role in supporting the nerve fibers.

A nerve fiber is defined as an axon and its Schwann cells, with or without a surrounding myelin sheath. The axon is the conducting cylindrical continuation of the cell body of the neuron, which is located in the spinal cord, dorsal root ganglia, or autonomic ganglia. The axon, which may be very long, reaches the end-organ (sensory receptors or neuromuscular junction) and contains axoplasm, which itself contains proteins and cytoskeletal elements[71]. The axoplasm is continuously produced and axonal transport mechanisms[16,71], including a bidirectional fast transport and a slow anterograde transport, allow movement of elements within the axon.

Each axon, along their longitudinal extent, is in proximity to Schwann cells. For some axons, the membrane of each Schwann cell wraps concentrically around each of their segments, providing a lipoprotein coating, known as myelin[43]. The myelin is thinner as the edge of one Schwann cell approaches that of another, forming areas called "node of Ranvier", which allow ionic exchanges between the axoplasm of a nerve fiber and the intercellular space[71]. These exchanges permit salutatory conduction of a nerve action potential, increasing the speed of nerve conduction of these myelinated fibers. Other axons are enveloped by the membrane of a Schwann cell, but without the formation of a significant lipoprotein sheath[101]. These fibers, which are unmyelinated, transmit impulses continuously along the axon and therefore have a slower conduction velocity[71]. Schwann cells, contrary to other intraneural cells such as fibroblast or mast cells, have the ability to form a basal lamina which surrounds them and all their contents[71].

The nerve fiber as a whole, i.e. the axon and its Schwann cells, is of variable size: unmyelinated nerve fibers are between 0.15 μm and 2.0 μm in size and myelinated nerve fibers are between 1μm and 20 μm in size[43]. The larger fibers conduct afferent and efferent motor signals, as well as afferent signals involving touch, pressure, and some painful sensations[71]. Smaller fibers are efferents for the autonomic system and afferents for temperature and most pain perceptions[71].

The supporting tissue of peripheral nerves, from the outside in, consists of the epineurium, the interfascicular epineurium, the perineurium and the endoneurium. These elements provide the framework for the nerve fibers. The amount of supporting connective tissue in peripheral nerves varies, both from nerve to nerve as well as from level to level within a given nerve[127]. In addition, some describe another supporting element external to the epineurium, the mesoneurium[71,77]. In normal peripheral nerves, this layer, filmy and transparent, secures the nerve to adjacent structures such as tendons, vessels, muscles, and fascial planes[71].

The epineurium is the outer supportive tissue of peripheral nerves. It is composed of longitudinally-oriented collagen fibrils forming a loose areolar connective tissue containing a vasa nervorum[43]. The epineurium is continuous with the interfascicular epineurium which is the supporting tissue between and surrounding the fascicles[71]. The

interfascicular epineurium is not as compact as the epineurium proper and its volume is variable depending on the nerve and level[71].

The perineurium is the supportive layer surrounding each fascicle. It is formed by flat perineurial cells and oblique, circular, and longitudinal collagen fibrils[43,71]. The perineurial cells, similar to Schwann cells, possess an outer and inner basement lamella[43]. The outer basement lamellae may play a role in transport of molecules[40], whereas the inner lamella provides an effective blood-nerve barrier with its thigh junctions between contiguous cells[82]. The perineurium is also the major source of the nerve tensile strength[71].

The endoneurium is the connective tissue around each myelinated nerve fiber and around each group of unmyelinated or poorly myelinated fibers within the perineurium[71]. Formed by small-diameter collagen fibers parallel to the axon axe and fine elastic fibers, it contains a few histiocytes, mast cells, and capillaries[71]. The endoneurium protects the axons when the nerve is mildly elongated or stretched[71].

The peripheral nerve vascular supply includes two integrated but functionally independent microvascular system[77], i.e. an extrinsic and an intrinsic vascular system. The extrinsic system is composed of segmentally-arranged vessels, which originate from adjacent small muscular and periosteal vessels, as well as from nearby large vessels[77]. These vessels, once they reach the epineurium, divide into ascending and descending branches and anastomose with the intrinsic system. The intrinsic peripheral nerve vascular system consists of vascular plexuses within the various neural supportive tissues[76,77]. The epineurial vessels are abundant, longitudinal, and consist of arterioles and venules[2,77]. They communicate with the plexuses within the perineurium and endoneurium[71,77] by obliquely piercing through the perineurium[77]. Within the endoneurium, the plexus consist generally of 2 to 6 capillaries per fascicles. These endothelial microvessels have thigh junctions and serve as another blood-nerve barrier[77].

2.2 Pathophysiology of Nerve Injury and Physiology of Nerve Regeneration

A variety of mechanisms can cause peripheral nerve injuries that differ in their outcome and potential for regeneration. The main mechanisms of nerve injury are laceration, traction, compression, gunshots (generally causing contusive and stretch injury), ischemic, electrical, thermal, irradiation, injection, and iatrogenic injuries. Peripheral nerve injuries are classified according to the extent of tissue damage. Two main classifications are used. The classification proposed by Seddon[115] consists of three types of nerve injury depending on the degree of injury: neurapraxia, axonotmesis, and neurotmesis. In neurapraxia, a conduction block is present, but there is no loss of axonal continuity. Neurapaxia is likely a biochemical injury and may involve a segmental demyelination[71]. By comparison, axonotmesis involves the disruption of the axonal and myelin continuity, but the connective tissue framework of the nerve is preserved. Finally, in neurotmesis, the axons, but also the nerve's connective tissues are completely disrupted. Sunderland proposed another classification consisting of five types of injury[125]. Grade I is a neurapraxic injury in which there is a conduction block. Grade II injuries are pure axonotmetic lesions without involvement of the connective tissues, i.e. only the axon and myelin are disrupted. Grade III lesions are more severe injuries in which there is a mixture of axonotmetic and neurotmetic lesions; the axon is disrupted, but also the endoneurium. In grade IV lesions, the axons, endoneurium, and perineurium are disrupted, but the epineurium is preserved. In grade I to IV lesions, the nerve remains in gross continuity. Grade V nerve injuries are transecting injuries with, by definition, interruption of all connective tissue layers. Mackinnon has proposed an additional type of injury, grade VI, consisting of a mixed lesion[84].

After a neurapraxic or grade I nerve injury, the affected segment will be remyelinated and there will be a resolution of the biochemical injury. Usually, recovery occurs within days to weeks. When the nerve injury was severe enough to disrupt the axon (i.e. axonotmesis, neurotmesis, or grade II to V lesions), Wallerian degeneration will occur distal to the lesion. The sectioned axon and its surrounding myelin degenerate along its entire length distally. The Schwann cells are activated and, along with the macrophages, will eliminate all cellular debris through phagocytosis[113]. In this process, the distal basal lamina will remain intact and the Schwann cells will proliferate in

anticipation of axonal regeneration[71]. Proximal to the lesion, the axon and myelin also degenerate for a short distance (approximately 1cm or 1 node of Ranvier).

When its axon is interrupted, the cell body of the neuron undergoes chromatolysis[43]. This consists of an increased cytoplasm volume, displacement of the Nissl substance to the periphery of the cell, and an increased metabolic rate[71]. These changes, beginning 4 days after the injury and peaking at 20 days, are primarily due to an increase in ribonucleic acid (RNA) and associated enzymes[71]. However, it is possible that the RNA increase serves only as a marker for regeneration and not as an initiating event[35]. In any case, the increased RNA volume and activity persist until axon regeneration and maturation cease[71]; it provides the necessary polypeptides and proteins for axoplasm replenishment and axon reconstruction[71]. The slow component of axonal transport will carry the proteins, or "building blocks", to the advancing neurite. This transport occurs at a rate of 1-2mm per day[16] and mirrors the rate of nerve regeneration. These processes for axonal regeneration occur in most neurons. Occasionally however, in severe proximal injury to neural elements, retrograde damage and changes to the neuron can lead to neuronal cell death[71].

After degeneration of their distal part, each axon produces a growth cone, composed of multiple axonal sprouts[113]. These axonal sprouts are guided by the Schwann cells, now arranged in longitudinal bands (bands of Büngner) [43]. The basal lamina and the relatively structured tubular system of the degenerated nerve also help to direct axonal regrowth[71]. However, the disorganized proliferation of both the endoneurium and interfascicular epineurium in response to injury may force the regenerating nerve fibers to change pathways and/or divide[71]. The factors responsible for such fibroblast proliferation and subsequent collagen alignment after injury are poorly understood[71]. This intraneural scarring, depending on its severity, may result in distal stump axons of fine caliber and of relatively poor myelination[71]. Only the axons reaching a distal end-organ receptor will mature and get myelinated; the others will either degenerate or fail to mature[109].

The growth cone depends on Schwann cell contact for elongation as well as for guidance[48]. The local environment also provides adhesiveness, access to laminin and fibronectin, as well as other factors that favor regeneration[49,71]. Trophic or growth factors

that originate from Schwann cells proximal and distal to the injury site also seem important[71]. A proximal stump, separated by a distance from the distal stump, will preferentially grow towards it rather than to non-neural tissue[79]. There is also evidence for neurotrophic interactions between regenerating axons and distal inputs such as muscles[71].

Functional motor recovery in nerve injury is limited by the time it takes for axonal regeneration to occur. When muscle is denervated, its structure changes histologically: the muscle fibers kink and their cross-striations decrease[128]; clinically, the muscle atrophies. With continued denervation, fibrosis will gradually replace the muscle and after 2 years without innervation, the muscle is usually totally replaced by scar tissue and/or fat[46].

Regeneration will occur at a speed of 2 to 5 mm/day in humans[113] and will lead to reinnervation if the nerve sheet (epineurium and/or perineurium) is intact. However, if there is discontinuity of the epineurium and/or of the perineurium (as in neurotmesis) or if the extent of endoneurium and interfascicular epineurium scarring in response to injury is too important, regeneration is impaired and the process will lead to the formation of a neuroma.

Clinically, nerve injuries lead to motor and/or sensory deficits. Injury to nerves of the upper extremities will lead to loss of motor function of the arm and/or hand, whereas lesion to nerves of the lower extremities may impair walking. Motor function loss may be accompanied by sensory deficits. The loss of pain and temperature sensation increases the risk of further traumatic injury to the denervated limb. Development of pain is another important complication of nerve injury and may be secondary to denervation and/or to the formation of painful neuromas.

2.3 Surgical Strategies for Nerve Repair

Surgical treatment of nerve injuries is based on the mechanism of injury, the extent of tissue damage (classification of nerve injury), the completeness of the lesion, and the presence or absence of spontaneous regeneration. In neurapraxic (grade I) lesion, spontaneous recovery will occur and no surgery is necessary. In neurotmetic (grade V) lesions, recovery is not possible without a surgical repair. In axonotmetic (grad II-IV) lesions, the regeneration will depend on the degree of supportive tissue injury. Some lesions will spontaneously recover and others will necessitate a surgical intervention. Interventions aimed at the repair of nerve lesions need to ideally occur within 6 months of the injury; earlier repair generally leads to better results. After this initial period, the chances of adequate functional recuperation decrease exponentially; if after repair the regenerated axons reach the neuromuscular junctions more than 2 years after the injury, the muscle atrophy will not be reversible.

In the presence of an acute nerve laceration without retraction or loss of substance, the nerve stumps are approximated using epineurial or fascicular microsuturing without an intervening nerve graft. However, when a gap is present between the nerve stumps, either due to retraction of the disrupted nerve, loss of substance, or necessity to remove a neuroma-in-continuity, the use of a nerve graft or nerve guide is necessary. Other nerve repair options include the transfer of a functioning nerve or fascicle to the injured nerve, especially when a functioning proximal nerve stump is not available for grafting. Occasionally, a nerve graft may be necessary to bridge the nerve transfer.

The autologous nerve graft is the current "gold standard"[66] to bridge nerve defects, because of its physiological architecture and viable Schwann cells. Various autologous donor nerves are available, the most common being the sural nerve[60]. Other donor nerves include the medial cutaneous nerve of the arm, superficial sensory branch of the radial nerve, and the internal saphenous nerve. However, autografts have several limitations including their limited availability, the associated donor site morbidity (scar, loss of sensation, formation of painful neuroma, potential for neuropathic pain) and often their inappropriate diameter[60,119].

2.4 Nerve Tubulization in the Treatment of Nerve Injury

Entubulation, also known as nerve tubulization, is a technique in which the nerve ends, when an intervening gap is present, are enclosed within a tube made of biological or synthetic materials[104]. The first tubulization repairs were attempted at the end of the nineteenth century[142], had disappointing results[126], and were not further utilized. In the 1980s, the concept was reintroduced mainly as an investigational tool of nerve regeneration[31]. These first tubes, made of silicone, could support axonal regeneration across a 1-cm gap in the rat sciatic nerve model[78]. After these first results, other nerve tubes composed of synthetic materials were developed as an attempt to find an alternative to autografts. Two main categories of nerve tubes were studied: the non-biodegradable materials[4,86,111,134,146] and the biodegradable materials[29,38,51,107,114]. More recently, tissue-engineering approaches are being explored. These include the manipulation of tissues in order to mimic the properties of the nerve structure and the enrichment of biological or synthetic tubes with various elements in order to enhance axonal regeneration. However, these newer techniques have not yet been applied in clinical studies and remain at the development stage. As multiple variables can impact the success and utility of nerve tubes for axonal regeneration across a nerve gap, these different strategies have limitations (table 2.1) and there is still no alternative to autograft. The success of the nerve tubes from non-biodegradable and biodegradable materials in human nerve regeneration as well as the ideal characteristics for nerve tubes are discussed below.

Table 2.1

Overview of Nerve Tubulization Strategies

Strategies		Examples	Variables impacting success and utility of nerve guidance tube for nerve regeneration											Main problems
			Biocompatible	Bioresorbable	Compression, foreign tissue reaction	Connective tissue	Guidance of nerve fibers	Vascularization	Growth factors	Properties for suturing and repair: Flexibility	Resistance	Size	Availability	
Biologic		Autografts	+	-	-	-	±	±	±	+	+	-	±	Limited availability and sizes
		Blood vessels: Arteries (early attempts) Veins	+	-	±	-	±	±	±	+	+	±	±	Limited support over long gaps
		Skeletal muscles ± pre-degeneration	+	-	±	±	±	±	±	±	±	+	+	Limited results
Synthetic	Non-biodegradable	Silicone Polytetrafluoroethylene (PTFE)	+	-	+	-	±	-	±	±	+	+	+	Compression reported over time
	Biodegradable	Polyglycolic acid (PGA) Poly-lactic-glycolic acid (PLGA) Poly-L-lactide-caprolactone (PLCL)	+	+	±	-	±	-	±	+	+	+	+	Limited support over long gaps
Tissue-engineering approaches	Manipulation of different tissues/organs	E.g.: Nerve-vein-combined graft Vein-muscle-combined graft Collagen	+	+	±	-	±	-	±	+	+	±	+	Limited support over long gaps
	Enrich biological or synthetic tubes with various elements	Growth factors Schwann cells	+				±	-	+					New strategies, no clinical studies yet
	Present thesis attempt		+	±	-	-	±	+	±	+	+	+	+	Attempt at a new strategy

2.4.1 *Nerve Tubes from Non-Biodegradable Materials*

Nerve tubes made of non-biodegradable materials include silicone and polytetrafluoroethylene (PTFE) tubes. A prospective randomized clinical study in human was done to evaluate silicone nerve tubes for the repair of small defects of the ulnar or median nerve just proximal to the wrist. Both at the 1- and 5-year follow-up, no significant difference in outcomes were found[80,81]. However, silicone tubes have been widely criticized[27,91] and are now out of favor. Over time, they may lead to nerve compression[17,26,27,91,92] and their removal is occasionally required after nerve regeneration[17]. Because they are non-biodegradable, they can also induce toxic levels of metabolic degradation products[31,66]. Only a few clinical reports have been published on the use of PTFE tubes [103,105,124].

2.4.2 *Nerve Tubes from Biodegradable Materials*

The nerve tubes made from biodegradable materials are the most promising synthetic nerve grafts. They include tubes constructed from resorbable polymers, such as polyglycolic acid (PGA), poly-lactic-glycolic acid (PLGA) and poly-L-lactide-caprolactone (PLCL). Presently, five biodegradable nerve guide implants are approved for use in humans: *Neurotube* (PGA), *Neuro-Matrix* and *Neuroflex* (collagen), *Neurolac* (PLCL), *Neuragen* (collagen), *SaluBridge* (polyvynil alcohol hydrogel)[112]. However, they are mainly used in the repair of sensory nerves of small caliber and with short nerve gap distances (less than 3cm), such as digital nerves.

Only a few randomized studies and large series have been published on the use of these tubes in humans. PGA nerve tubes are reported to have comparable results to conventional nerve repair for the repair of small digital nerve gap lesions in one randomized[141] study and two large series[9,83]. However, the data should be interpreted with care and these results may not be applicable to the treatment of other nerve lesions. PLCL nerve tubes were also assessed in a randomized multicenter trial[14] and there was no significant differences in sensory recuperation from direct coaptation repair. Again, this study focused on digital nerve repair, and the results may not be applicable to other nerve lesions.

2.4.3 *Ideal Characteristics for Nerve Tubes*

The design of nerve tubes for the treatment of nerve injury demands that the container be distinguished from the contents. The outer surface of the nerve tube, i.e. the container, forms a barrier that prevents ingrowth of fibroblasts into the nerve gap. The contents of the nerve tube supply a surface area onto which migrating Schwann cells and growth cones from the amputated nerve ends can preferentially attach[129]. The characteristics of each of these different components influence the nerve regeneration process and need to be taken into consideration. Previous studies indicate that certain characteristics are important in the design and development of a nerve guidance tube.

The nerve tube itself, i.e. the container, must first be permeable. Although the exact degree of permeability required to optimize nerve regeneration is unknown, multiple studies confirmed the importance of permeability[3,63-65,69,107,137]. Inadequate permeability may impair the cells (macrophages and leukocytes) and molecules (fibrin and fibronectin) involved in the formation of the fibrin matrix to enter the site of regeneration[31]. On the other end, excessive permeability, such as macropores, may allow neurotrophic factors to diffuse outside of the nerve tube and may lead to a disorganized matrix[31]. The inner texture of the nerve tube as well as its dimension will also influence the formation of the fibrin matrix[111], necessary for nerve regeneration. In nerve tubes with a smooth surface (e.g. silicone tubes), the longitudinal matrix will coalesce and form a free-floating nerve cable, whereas with rough surfaces, the matrix will disperse and fill the lumen of nerve tube[44]. The nerve tube should also be flexible, but able to resist deformation (elongation, breaking, or kinking) and be strong enough to hold a suture[31]. In biodegradable tubes, the degradation process should be studied; degradation products may cause swelling by increasing the osmotic pressure[29,30], may be toxic, may interfere with the regeneration process, or may affect the porosity and tensile properties of the tube over time[31]. Finally, a transparent nerve tube is preferred; it facilitates suturing and accurate positioning of the nerve stumps within the tube[31]. The nerve tube should be able to withstand the sterilization procedures, without compromising its properties[31].

There is more and more evidence that a simple tubular structure cannot support optimal nerve regeneration[12,33]. A consensus emerged that bridging long peripheral nerve gaps will require nerve tubes with an internal architecture that will promote regeneration by supporting and directing axonal migration[12,66]. When nerve gaps are short (≤10mm in

rats), a fibrin cable will form across the gap[36,59] and allow Schwann cell infiltration, formation of bands of Büngner, and axonal regeneration[12]. However, in large nerve gaps (>15mm in rats), the formation of the fibrin cable and of the bands of Büngner is compromised; exogenous support is necessary[5,12,131].

To enhance regeneration across peripheral nerve gaps, the contents of the nerve tubes can be manipulated. First, a growth permissive substrate, or an internal framework, can be added. Different strategies are being explored, from the filling of a tube with an alginate gel to the addition of microtubes, microfibers, or nanofibers within the tubular biomaterial[12,96,97]. Changes in the structural scaffold (oriented versus non-oriented) may influence the regeneration response of the growth cones[12]. In general, neurite extension is better on 2-dimensional surfaces than when the neurons are embedded in 3-dimensional substrates[11,42,122]. An ideal internal framework may consist of distributing 2-dimensional surfaces, which would support the regenerating axons, in a 3-dimensional space[12]. However, the total cross-sectional area that is physically available to the regenerating nerve must be considered; the ideal scaffold would maximize the guidance cues, while minimizing physical obstruction to the regeneration[12].

Another strategy is to supplement the nerve tubes with neurostimulatory extracellular matrix proteins or peptides, such as laminin-1 or laminin-1 fragments. Extracellular matrix proteins on the tube's inner surface may favor axon adhesion and growth[39]. Anisotropic distribution of these peptides may also enable faster or better regeneration[12]. For example, growth cone extension across a laminin-1 gradient (even if down a gradient) is superior to growth across uniformly distributed laminin-1[1,32]. Other strategies include the incorporation of trophic factors, such as basic fibroblast growth factor (bFGF), nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF), or of glial or other supportive cells, such as Schwann cells or stem cells[47,74,96,97]. These methods could also potentially enhance regeneration.

Finally, the design of nerve tubes, especially if they are to be of large diameter, should address their vascularization potential. For nerves and nerve grafts of small caliber (<2mm), passive diffusion is sufficient for oxygen and nutrients supply as well as for toxin removal. However, larger nerves require a vascular supply and axons are very

sensitive to hypoxia. The vascularization of nerve tubes is a major obstacle to reinnervation for potentially large size nerve guides.

CHAPTER 3 – MATERIAL AND METHODS

CHAPTER 3. MATERIAL AND METHODS

This material and methods section is divided in 3 separate sections, in order to address the 3 specific hypotheses of this thesis.

3.1 Development of a Tissue-Engineering Approach to Produce Completely Biological Nerve Guidance Tubes from Cultured Human Cells

The development of a tubular biomaterial from human cells to support nerve regeneration requires the use of innovative methods. Since both nerve repair and vascular repair require the development of a tubular structure with good mechanical resistance, the methods that will be used are based on a successful technique able to reconstruct blood vessels in-vitro[75]. This original and effective approach was developed by a team of the LOEX and this project is done in collaboration with their laboratory, under the supervision of Dr. François Berthod. However, as a simple tubular structure cannot support optimal nerve regeneration, the developed approach needs to be modified and adapted.

3.1.1 *Previous Achievements of the LOEX*

L'Heureux et al[75] were able to produce completely biological tissue-engineered human blood vessel. This technique consisted of: 1) smooth muscle cells and fibroblasts cultured in vitro until the production of a cellular sheet; 2) the cellular sheet is then wrapped around an inert tubular support of a diameter defining the dimension of the lumen of the tube; and 3) a maturation period in-vitro allows the sheets to fuse together producing a tube with excellent mechanical properties[75]. This method offers possibilities of producing living or lyophilized tubes. Lyophilized tubes are easier to manipulate and are decellularized; they can therefore be made from heterologous cells.

This thesis was based on the technique described above and on experiments recently performed at the LOEX for the production of nerve guides from cultured human cells. In those recent studies, fibroblast sheets were used to produce a nerve guide from cultured human cells. These tubes were lyophilized and used as hollow tubes or filled

with a chitosan-collagen gel. The chitosan-collagen gel was used to provide an internal architecture for the support of nerve regeneration. The mechanical properties of these tubes were adequate to allow suturing. The techniques (Figure 3.1) involved in the production of these tubes are also utilized in this project and are described below.

The present project first goal is to replace the chitosan-collagen gel to produce a biological nerve guidance tube produced *completely* from cultured human cells, with an internal architecture able to support nerve regeneration. Previous limited attempts at rolling the fibroblast sheets without an inert tubular support to produce tubes with an internal architecture have been tried. However, the structure and characteristics of these tubes were irregular, variable, and non-reproducible. This project will explore different methods to produce tubes with an internal architecture, all from cultured human cells.

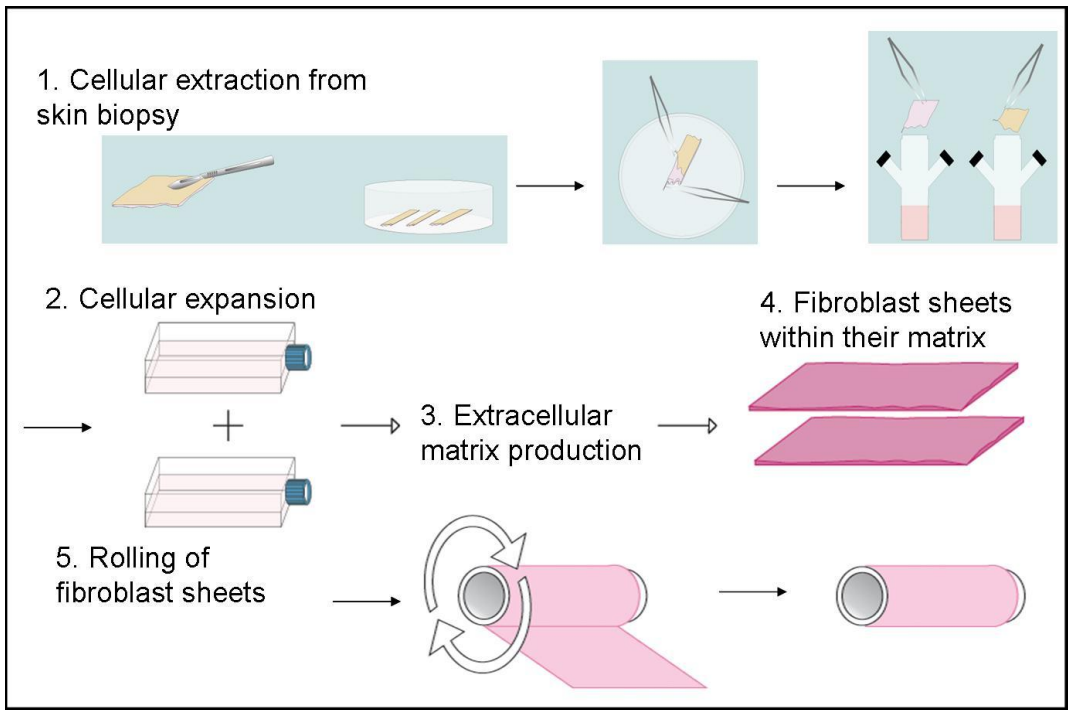

Figure 3.1 Formation of Hollow Nerve Tubes from Fibroblast Cells

Artistic illustration of the technique used to produce hollow nerve tubes from fibroblast cells. The fibroblasts are extracted from the skin (1), expanded (2), and cultured in ascorbic acid (3) until the production of a fibroblast sheet (4). The fibroblast sheets are rolled around an inert tubular material (5) to produce hollow nerve tubes. Image from Jean Dubé, Loex, used and adapted with permission

3.1.2 *Human Fibroblast Cells*

Human fibroblasts from internal frozen cell line banks (available at the LOEX) were used for the production of the nerve guidance tubes. These banks were created by cellular extraction of fibroblasts from normal adult skin specimens obtained, with patients' consent, at time of breast reduction surgeries.

The method of cellular extraction used to isolate and culture human skin fibroblasts was previously published[75] and is briefly described here. Small skin fragments were floated in a 500 μg/ml thermolysin solution in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer at 37ºC for 2 hours. The dermis was then separated from the epidermis and incubated in a solution of collagenase (200 U/ml in Dulbecco's modification of Eagle's medium (DME)) for 20 hours at 37ºC. After centrifugation, fibroblasts were plated into tissue flasks in standard medium at a density of 10^4 cells/cm^2. The cultures were maintained at 37ºC in a humidified atmosphere (92% air and 8% CO_2). The fibroblasts were frozen in liquid nitrogen in a freezing media containing dimethyl sulfoxide (DMSO) for cryoprotection.

The fibroblasts used for all experiments were from frozen bank of healthy human subjects between the ages of 18 and 38. Various fibroblast cell lines (n=4) were used for the preliminary trials, after which the rest of the experiments were performed with two different cell lines. The fibroblasts were used between passages 3 and 7.

3.1.3 *Production of Human Fibroblast Sheets*

To induce extracellular matrix formation and the production of fibroblast sheets, fibroblasts were cultured in standard culture medium supplemented with 50 μg/ml of sodium ascorbate (Sigma) in 25 or 75 cm^2 culture flasks. The medium was composed of DME, supplemented with 10% fetal calf serum, and antibiotics (100U/mL of penicillin G and 25 μg/ml of gentamicin). The culture medium was changed 3 times per week. After approximately 30 days of culture in a humidified atmosphere (92% air and 8% CO_2), sheets composed of the fibroblasts and their extracellular matrix were formed. These sheets could be manually peeled off the culture flasks.

3.1.4 *Production of Hollow Nerve Tubes*

The fibroblast sheets produced with the technique above, in 25cm^2 culture flasks, were manually peeled off the culture flasks and wrapped around an inert tubular structure, producing a cylinder of concentric sheet layers. These concentric fibroblast sheets were then matured in culture medium for another 15 to 20 days, with culture medium changes 3 times a week. The nerve tubes were then decellularized, using repeated cycles of washing with water and drying, and lyophilized. Lyophilization was done in a Genesis 12EL vacuum lyophilizer (Virtis, Gardiner, NY). Once lyophilized, the tubes were stored in the dark at 4ºC until utilization.

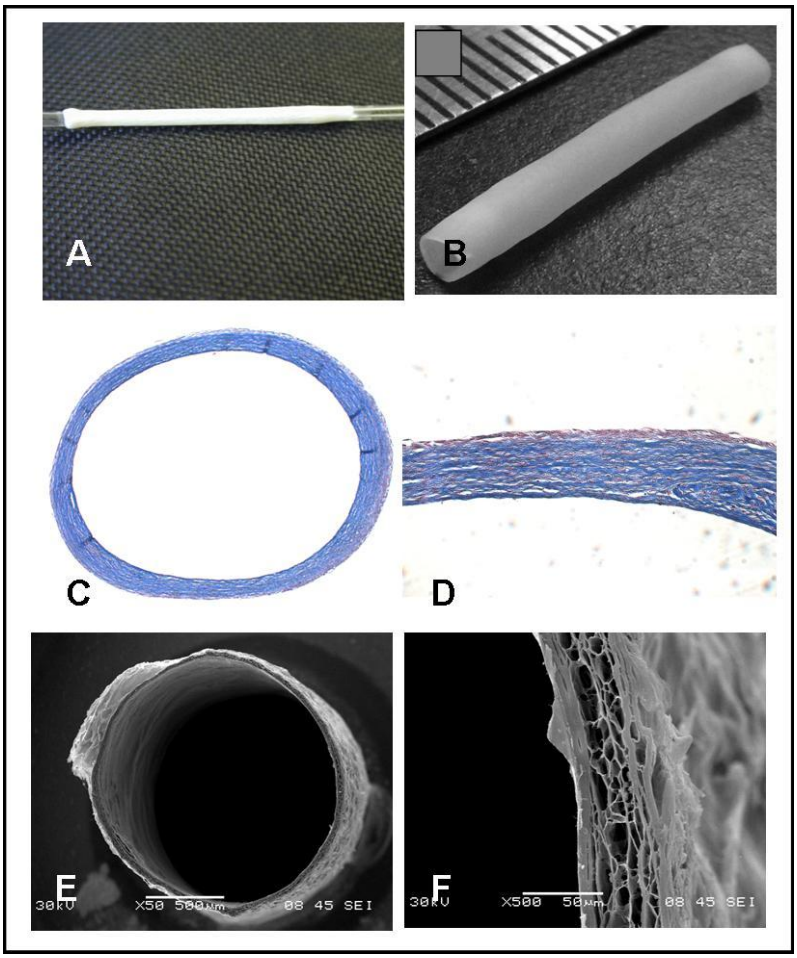

Figure 3.2 Hollow Nerve Tubes

Lyophilized hollow tube is seen on a glass micropipette after lyophilization (A) and once the glass micropipette has been removed (B). Microscopic appearance of the hollow tube; the concentric layers of the fibroblasts sheets are well seen (C, D). Surface electron microscopy image of the lyophilized hollow tube (E, F). Image from René Caissie, LOEX, used with permission.

3.1.5 *Production of Hollow Nerve Tubes Filled with Chitosan-Collagen Gel*

The chitosan-collagen gel was prepared by mixing type I, III bovine collagen (Laboratoire Perouse Implant, Chaponost, France) with chitosan II (SADUC, Lyon, France) dissolved in 0.1% acetic acid.

The liophylized hollow nerve tubes produced as described above, were first rehydrated in water. The tubes were then filled with the chitosan-collagen gel, closed, and lyophilized using a Gensis 12EL vacuum lyophilizer (Virtis, Gardiner, NY) for 40 hours. Once lyophilized, the tubes were stored in the dark at 4ºC until utilization.

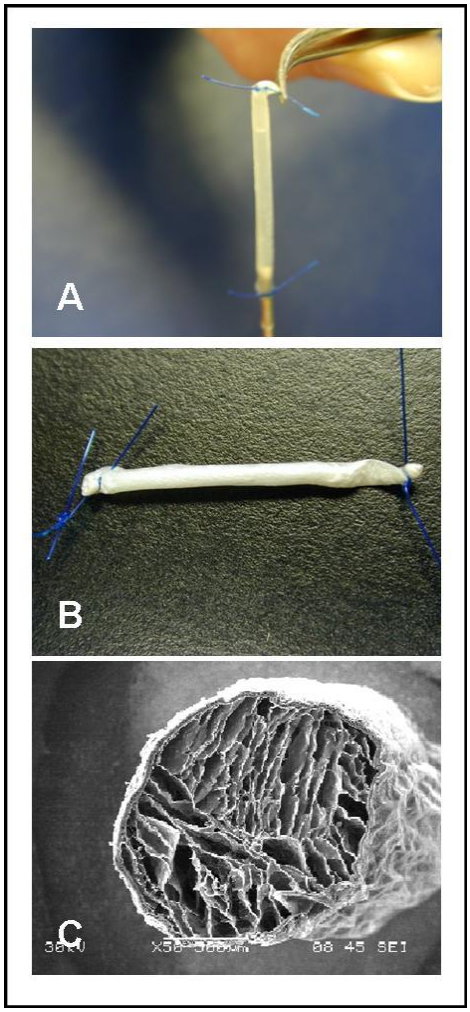

Figure 3.3 Hollow Nerve Tube Filled with Chitosan-Collagen Gel

A hollow tube is seen after being filled with the chitosan-collagen gel before(A) and after(B) lyophilization. The electron microscopic appearance of the hollow nerve tubes with chitosan-collagen gel filling is shown (C). Image from René Caissie, LOEX, used with permission.

3.1.6 *Production of Filled Tissue-Engineered Nerve Tubes*

As previous attempts at rolling the fibroblast sheets without an inert tubular support produced tubes with an irregular, variable, and non-reproducible structure, different methods were explored. To experiment different assembly methods, fibroblast sheets composed of fibroblasts and their extracellular matrix were produced as described in the previous section. The fibroblasts were cultured in culture flasks of 25 and 75 cm^2 to evaluate both sheet formats. After approximately 30 days of maturation, the sheets could be manually peeled off the culture flasks and manipulated.

Different techniques to roll the fibroblast sheets on themselves, without the use of a central tubular structure, were tried, including the use of tweezers and sutures. All attempts were performed using a sterile technique within a laminar flux cabinet in order to avoid microorganism contamination. These techniques were based on the idea that a fibroblast sheet could potentially be rolled on itself, producing a cylindrical structure filled by concentric layers of cells in extracellular matrix. Regenerating nerve fibers could potentially migrate in between those layers, which would provide the necessary support for their regeneration.

The filled tubes obtained by the newly developed technique were matured under traction between 1 and 30 days in petri dishes in the standard culture medium. The culture medium was changed 3 times per week. After different maturation times, the specimens were fixed in histochoice, included in paraffin, cut in 5µm transverse section and stained with Masson's trichrome to evaluate the internal and external structure of the tubes. The reproducibility of the technique was evaluated qualitatively by histological appearance.

The ability to suture the constructed tubes was evaluated qualitatively with microsurgical technique under loop or microscopic magnification. The tubes were modified as described in the result section since they could not easily be sutured.

3.1.7 *Production of Rat Fibroblast Sheets*

In order to study the possibility that completely biological tissue-engineered nerve grafts could be created from autologous cells and be implanted as living nerve guides, the development of an animal model was necessary. As the rat sciatic nerve model is the most frequently used to test nerve regeneration, we attempted to produce rat fibroblast sheets that could be manipulated into tubes as described above for the human fibroblast sheets.

Rat fibroblast cell lines (Sprague-Dawley rat strain) previously extracted and available in the LOEX cell bank were used. The fibroblasts had been extracted with a protocol similar to the one used for human fibroblasts and described above.

Three cell lines were chosen and fibroblasts in first passage were used. The fibroblasts were first thawed and expanded. They were cultured in medium composed of DME, supplemented with 10% fetal calf serum, and antibiotics (100U/mL of penicillin G and 25 μg/ml of gentamicin). The medium was changed 3 times a week. The cultures were maintained at 37ºC in a humidified atmosphere (92% air and 8% CO_2). The fibroblasts were subsequently seeded in passage 3 and 4 into 25 and 75 cm^2 culture flasks for the formation of fibroblast sheets. After seeding, the standard culture medium was supplemented with 50 μg/ml of sodium ascorbate (Sigma). The fibroblasts were matured for a period of 30 to 45 days for the evaluation of the formation of extracellular matrix. The success in formation of fibroblast sheets from rat fibroblasts was assessed qualitatively and the cellular cultures were modified as needed.

3.2 Production of Nerve Guidance Tubes with a Capillary-Like Network from Cultured Human Cells

3.2.1 *Formation of a Capillary-Like Network*

The ability to create a capillary-like network within the newly designed tissue-engineered nerve guides was evaluated. To evaluate this, endothelial cells were seeded on fibroblast sheets which then were rolled into filled tube with the newly developed technique.

Human umbilical vein endothelial cells (HUVEC), which are known to form capillaries in-vitro in reconstructed dermis[15,133], were first used. The cells were taken from an internal frozen cell line bank. These HUVEC were obtained from healthy newborns umbilical cords by enzymatic digestion with 0.250 μg/ml thermolysin (Sigma). The extraction method has previously been described[133]. The HUVEC cells were thawed and expanded. They were plated on gelatin-coated tissue culture flasks, and cultured in EGM-2 medium (Clonetics, Walkersville, MD). The medium was changed 3 times a week.

HUVEC were seeded onto fibroblast sheets (30 days of maturation) at the sixth passage. The seeded sheets were cultured in EGM-2 medium supplemented with 50 μg/ml of sodium ascorbate (Sigma) and maintained at 37ºC in a humidified atmosphere (92% air and 8% CO_2). After 3 days of co-culture, the fibroblast sheets seeded with the endothelial cells were rolled into filled tubes with the method developed previously. They were matured in petri dishes with EGM-2 medium containing sodium ascorbate. The endotheliazed filled tubes were kept in culture for various maturation times (4, 14, and 31 days). At each time point, specimens were taken. Part of the specimen was fixed in Histochoice's solution (Armesco, Solon, OH), embedded in paraffin, cut in 5μm-thick transverse sections, and stained with Masson's trichrome. The specimens were examined for the presence of capillary-like structure. Part of the specimens was also frozen in O.C.T. (optimal cutting temperature) cryoembedding media (Somengen, Edmonton, Canada). Cryosection of 5μm were used for immunofluorescence studies to assess the presence of endothelial cells. A mouse monoclonal anti-human platelet endothelial cell adhesion molecule-1 (PECAM-1) antibody (Chemicon, 1/800 dilution) revealed with

Alexa 594-conjugated goat anti-mouse IgG (1/500 dilution) was used. The second antibody was mixed with Hoescht (1/100 dilution) [Sigma] to observe the cell nuclei. A few specimen were double-labeled with a mouse monoclonal anti-PECAM-1 antibody (Chemicon, 1/800 dilution) revealed with Alexa 594-conjugated goat anti-mouse IgG (1/500 dilution) and with a rat monoclonal anti-laminin antibody (Immunotech 8, 1/100 dilution) revealed with Alexa 488-conjugated goat anti-rat IgG (1/200 dilution). The fourth antibody was mixed with Hoescht (1/100 dilution) [Sigma] to observe the cell nuclei.

3.2.2 *Characterization of the Growth and Formation of a Capillary-Like Network*

To better characterize the growth and formation of the capillary-like network within the tissue-engineered nerve tubes, HUVEC transfected with green fluorescent protein (GFP) were used (HUVEC-GFP). A cell line of HUVEC-GFP given by an external laboratory (Jeffrey A. Medin, Division of Experimental Therapeutics, Ontario Cancer Institute, University Health Network, Toronto, ON, Canada) was utilized. These cells, with enhanced GFP (enGFP) expression, were engineered using a recombinant HIV-1-based lentiviral vector, LV/enGFP. Vesicular stomatitis virus glycoprotein-pseudotyped viruses were produced by transient co-transfection of 293T cells (ATCC) with plasmids pHR'-EF-GW-SIN, pCMVDR8.91 and pMD.G[145] using calcium phosphate precipitation[110]. At 24 and 48 hours following transfection, supernatant was collected, passed through a 0.45µm filter and applied to HUVEC cultures. Cells were transfected twice in the presence of 8µg/ml protamine sulfate.

The HUVEC-GFP were thawed from the cell line described above and expanded on gelatin-coated tissue culture flasks with EGM-2 medium (Clonetics, Walkersville, MD). The culture medium was changed 3 times a week. The HUVEC-GFP were seeded onto the fibroblast sheets (25 to 28 days after their formation) between the ninth and eleventh passage. Parallel HUVEC-GFP cells cultures on gelatin-coated culture flask were kept to monitor the presence of GFP expression in the endothelial cells over time.

Various initial seeding concentrations of HUVEC-GFP (between 0.3million cells /25cm^2 and 1.5million cells /25cm^2), various time of expansion of the HUVEC-GFP on the fibroblast sheets before rolling them into tubes (5 to 44 days), and various maturation

times once tubes were formed (1 to 30 days), were studied. The various conditions studied are shown in table 3.1. Usually, at least 3 samples of each condition were made. The capillary-like network was evaluated during culture on fibroblast sheets using a timelaps inverted microscope; once the tubes were constructed, the capillary-like network was characterized with confocal microscopy, using a Nikon C1 confocal microscope.

3.2.3 *Exploration of a Different Method of Fibroblast Sheet Assembly for Optimal Formation of Capillary-Like Network*

Finally, a different method of fibroblast sheet assembly before forming the filled tubes was designed. This new method of assembly was studied to determine the best approach to maximize the formation of a capillary-like network. The fibroblast sheets were produced, HUVEC-GFP were seeded at different initial concentration (0.3million cells/25cm^2, 0.6million cells/25cm^2, and 0.9million cells/25cm^2), and matured on the fibroblast sheets, all as described above. The fibroblast sheets with endothelial cells were assembled 7 days after the endothelial cell seeding. These assembled sheets were kept in culture for 9 to 15 days, after which they were rolled into tubes with the technique that was designed and is described in section x. The filled tubes were kept in culture for 1 to 30 days. The various conditions studied are shown in table 1. Usually, 3 sample of each condition were made. The capillary-like network was evaluated during culture on fibroblast sheets using a timelaps inverted microscope; once the tubes were constructed, the capillary-like network was characterized with confocal microscopy, using a Nikon C1 confocal microscope.

Table 3.1

Different Experiments using HUVEC-GFP

Experiments	Fibroblast sheet maturation	HUVEC-GFP seeded (per 25cm^2)	Maturation of HUVEC-GFP on fibroblast sheet		Maturation of construct after	
			Before assembly	Before rolling	Assembly before rolling	Rolling
1	26 days	0.3million		5 days		1 day
						4 days
						16 days
						30 days
2	25 days	0.3million		21 days		8 days
		0.6million				
		0.9million				
		1.2million				
		1.5million				
3	28 days	0.6million		44 days		8 days
4	26 days	0.6million		22 days		1 day
						4 days
						15 days
						30 days
5	26 days	0.9million		22 days		1 day
						4 days
						15 days
						30 days
6	28 days	0.3million	7 days	16 days	9 days	5 days
						15 days
						30 days
7	26 days	0.6million	7 days	22 days	15 days	1 day
						4 days
						15 days
						30 days
8	26 days	0.9million	7 days	22 days	15 days	1 day
						4 days
						15 days
						30 days

3.3 Study of Nerve Regeneration in Different Nerve Guidance Tubes Produced from Human Cultured Cells

The final step of this project is to study nerve regeneration in the different guidance tubes produced from human cultured cells. Previously, attempts were made to study nerve regeneration through the hollow tubes and hollow tubes filled with chitosan-collagen gel that had been developed. In those previous studies, the rat sciatic nerve model with Sprague-Dawley strain rats was used. However, the presence of autotomy prevented the completion of the studies. This problem, i.e. autotomy in the rat sciatic nerve model, needed to be addressed before the study of nerve regeneration in the different guidance tubes was possible. In this thesis, the rat sciatic nerve model was first modified to reduce autotomy rates. The previous study on nerve regeneration within the designed hollow tubes and hollow tubes filled with chitosan-collagen gel was repeated. Finally, the tissue-engineered nerve tubes designed in this study, the filled tubes, were tested in the rat sciatic nerve model.

3.3.1 *General Pre-operative, Operative, and Post-operative Protocol Used for Nerve Regeneration Testing in the Rat Sciatic Nerve Model*

The rats were generally received 1 week prior to surgery for acclimation. The surgery (Figure 3.4) was done under general anesthesia with inhalation of 1-2% isoflurane. Once anesthetized, the animals were placed in the prone position. The rat right gluteal area and right lower extremity was shaved and prepped 3 times with an alcohol-iodine solution. A postero-lateral incision along the axis of the femur was made, approximately 2 cm, extending from the gluteus muscle to the mid thigh. The right sciatic nerve was exposed from its exit from pelvic cavity to the level of its bifurcation into the nerve fibularis and nerve tibialis. The fascia overlying the nerve was carefully removed to expose the nerve surface with its epineurial covering maintained intact. A solution of 50:50 bupivacaine and lidocaine was put around the nerve before any further manipulation. The sciatic nerve was then sharply transected and a 10 to 15 mm segment was removed, proximal to the sciatic nerve bifurcation. The nerve was either repaired with an autograft, a nerve guide, or not repaired. For the autograft, the removed segment of sciatic nerve was reversed and used for repair. The reversal of the autograft was performed to reflect the clinical setting: the fascicles of the proximal nerve and of the

autograft are approximated but do not match. Performing two direct repair would likely lead to a result superior to an autograft; our goal was to compare the nerve guides with the clinical gold standard, the autograft. Other groups using the rat sciatic nerve repair model also perform a reversed autograft for positive control[37,47,102]. The reverse orientation is not thought to adversely affect orientation and may even decrease regenerating axon dispersion. 10-0 Ethicon sutures (2870G, needle BV75-3) were used for all nerve repairs. The muscle was reapproximated with interrupted sutures and the skin was closed with clips. For analgesia, the rats received an injection of 0.01 to 0.02mg/kg of buprenorphine thirty minutes before surgery and twice daily for 3 days post-operatively.

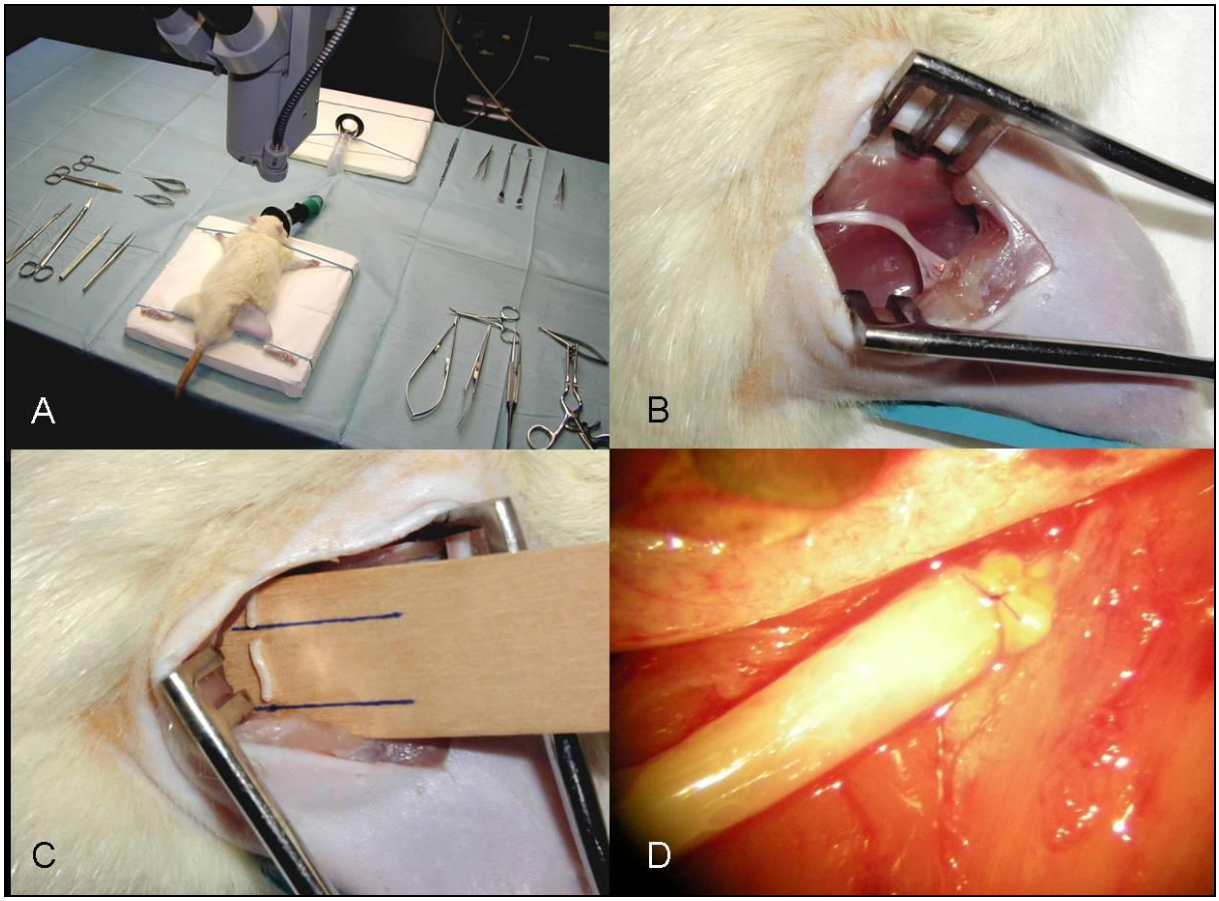

Figure 3.4 Surgical Technique for the Rat Sciatic Nerve Model

Surgery is done under general anesthesia with inhalation of isoflurane; the rat is positioned prone (A). A longitudinal incision along the femoral axis is performed and the sciatic nerve is visualized. The nerve gap is created with the chosen length. The nerve tube is sutured in place with 10-0 Ethicon sutures under microscope. Image in part (A,B,C) from René Caissie, LOEX, used with permission.

The rats were lodged 2 per cage and were allowed free access to water and standard rodent chow (Charles River). The rats were generally followed for 15 to 16 weeks as functional recovery occurs by that time after nerve repair, reaching near optimal recovery by 12 weeks[50]. They underwent manual physiotherapy daily to prevent contractures. Functional analysis was done pre-operatively and post-operatively at 3-week intervals.

Motor functional assessment consisted of walking track analysis and measurement of the sciatic function index (Figure 3.5). This motor function test provides a reliable and easily quantifiable method of evaluating the functional condition of the sciatic nerve[90] and is largely used as an indicator of sciatic nerve regeneration in the rat sciatic nerve model [6, 60,135]. It is based on measurements of footprint parameters of rats during their walk. The hindpaws of the rats were dipped in ink (blue stamp ink). The rats were then placed, one at a time, at the start of a corridor at the end of which is a black room. The rats instinctively walk toward the black box. A white paper was placed on the walking surface of the corridor at each trial. The prints left by the rats (at the time of brisk walking) were be used to calculate the sciatic function index, which reflects the motor functional recovery[6.135]. If needed, several trials were done. Prior to the surgical procedure, the rats were trained to walk in the corridor, and a baseline walking tract was recorded. The sciatic function index as described by Bain et al[6] was calculated using paired measurements of the print length (PL), toe spread (TS) (first to fifth toe), and intermediary toe spread (IT) (second to fourth toe). The measurements of the normal control left foot (NPL, NTS, NIT) and of the corresponding right experimental foot (EPL, ETS, EIT) were recorded for each rat at each time point. The measurements were then incorporated into the Bain-Mackinnon-Hunter sciatic function index (SFI)[6] (Figure 3.6).

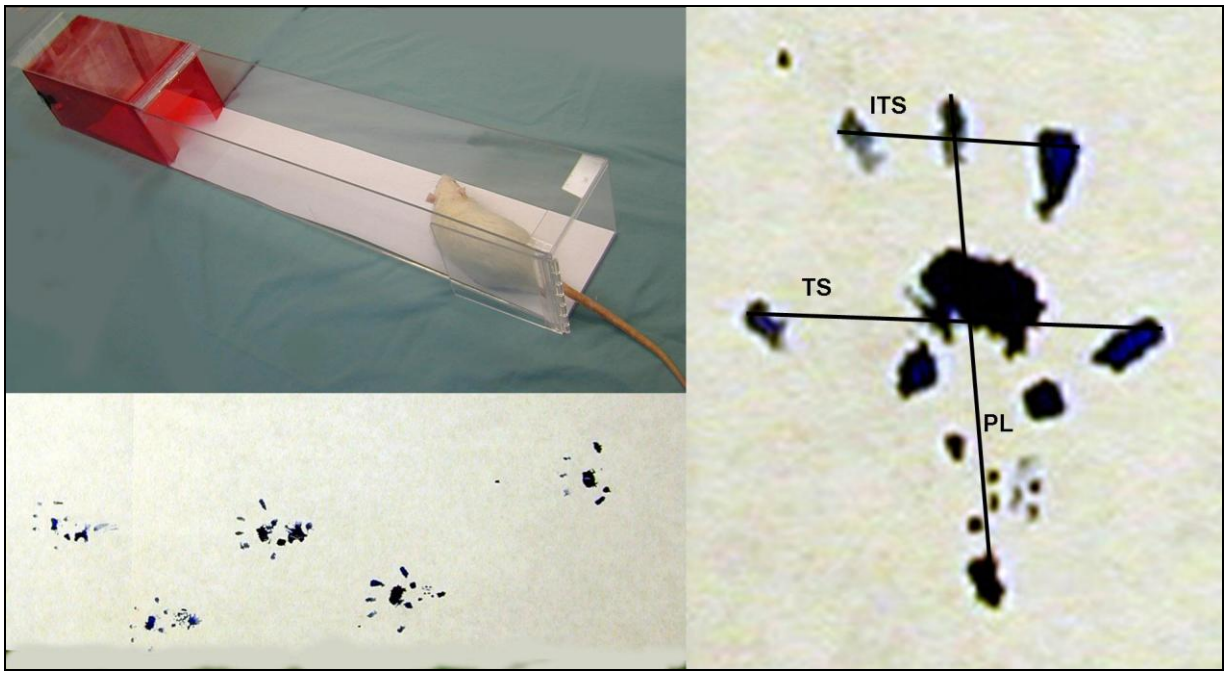

Figure 3.5 Walking Tract Analysis

Image from René Caissie, LOEX, used and modified with permission

$$SFI = -38.8\left(\frac{\text{Experimental PL}-\text{Normal PL}}{\text{Normal PL}}\right) + 109.5\left(\frac{\text{Experimental TS}-\text{Normal TS}}{\text{Normal TS}}\right) + 13.3\left(\frac{\text{Experimental ITS}-\text{Normal ITS}}{\text{Normal ITS}}\right) - 8.8$$

Figure 3.6 Bain-Mackinnon-Hunter Sciatic Function Index

Sensory functional recovery was assessed using transcutaneous sine-wave stimuli produced with a Neurometer (Figure 3.7). The cutaneous electrode was placed on the hindpaw plantar surface. Constant alternating current sinusoid waveform stimuli at intensities ranging from 0.01mA to 9.99mA (1 to 999) and at frequencies of 5Hz, 250Hz, and 2000Hz were used. The intensity of the sine-wave stimulus was gradually increased until the animal demonstrated discomfort characterized by torsion of the tail. For each frequency, three consecutive measures were taken from each hindpaw. The mean stimulus intensity at onset of discomfort was calculated for each hindpaw at each frequency. The 5Hz frequency selectively stimulates small unmyelinated C fibers (slow pain, nociception, and temperature), the 250Hz frequency selectively stimulates small myelinated Aδ fibers (mechanoreception, nociception, temperature, and fast pain) and the 2000Hz frequency stimulates selectively large myelinated Aß fibers (cutaneous touch and pressure)[77,99]. The percent change between the intensity at which the rat is responsive from the operated leg to the unoperated leg was calculated. This sensory functional assessment was performed at 3-week intervals during the post-operative period for the evaluation of hollow tissue-engineered tubes and hollow tissue-engineered tubes filled with chitosan-collagen gel. The Neurometer was not used to evaluate the lyophilized filled tissue-engineered tubes with and without a capillary network.

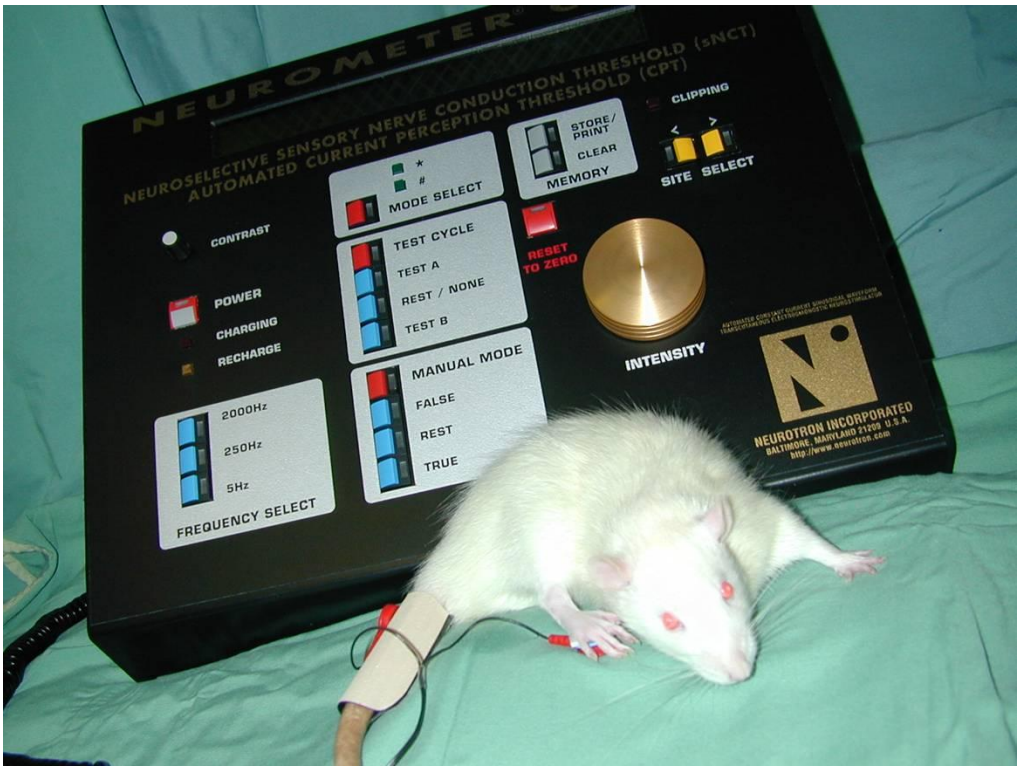

Figure 3.7 Neurometer (Image from René Caissie, LOEX, used with permission)

At the completion of the study period, the rats were sacrificed by mortal inhalation of CO_2. The surgical site was reopened and the repaired nerve was excised including the available proximal and distal nerve segments. Three sections were taken and labeled as proximal, graft, and distal sections. Each section were fixed in gluteraldehyde 2%, embedded in Epon, cut in semi-thin sections (1um) and stained with toluidine blue. Nerve regeneration was evaluated using the total number of myelinated axons. In addition, the weight of the gastrocnemius muscle was measured both from the right experimental and from the left normal lower extremity. Gastrocnemius muscle mass is proportional to the degree of sciatic nerve innervation[37] and provides evidence for functional activity of sciatic nerves. The relative gastrocnemius muscle weight (RGMW)[37,147], defined as the ratio of the gastrocnemius muscle from the experimental side to that of the normal side, was calculated and compared between the experimental groups.

For all numerical variables, means and standard errors (SEM) were computed. Differences between group means were evaluated using one-way analysis of variance (ANOVA). If the ANOVA demonstrated overall significance ($P < 0.05$), then specific group mean comparisons were performed for that variable using a post-hoc Tukey's significant difference test to correct for multiple comparisons and to maintain an overall alpha level of 0.05. P values less than 0.05 were considered statistically significant. Differences over time in numerical value were evaluated using matched pair analysis with the Wilcoxon signed-rank test. A P value of less than 0.05 was considered statistically significant.

3.3.2 *Adaptation of the Rat Sciatic Nerve Model to Reduce Autotomy*

Autotomy is defined as auto-mutilation and is assumed to reflect neuropathic pain[28,60]. In the rat sciatic nerve model, it consists of the auto-mutilation of their denervated paw (i.e. auto-mutilation of one or more toes and even of the whole hindpaw)[60,149]. When autotomy occurs, the functional assessment by walking track analysis is impaired. Moreover, in our institution, the rats need to be sacrificed for ethical reasons, leading to early termination of the experiment.

In the literature, different methods are used to avoid autotomy: medications such as peripheral sympathetic inhibitors (guanethidine)[139], tricyclic antidepressants (amitriptyline)[98], topical application of repellant[123], housing male rats with female rats[149], or the use different strains of rats[21,60]. It is reported that rats of Lewis strain have very low autotomy rates compared to other strains[21,50], including the Sprague-Dawley rats which were used in the previous attempts described above.

Based on the review of the literature, a preliminary study with 6 male Lewis-strain rats (Harlan Laboratories inc., Indianapolis, IN; between 250 and 300 grams) was done to evaluate their autotomy rates. A 10mm autograft was the chosen procedure, as in previous LOEX in-vivo experiments, this was the experimental group showing the most severe form of autotomy. In previous experiments performed at the LOEX, as well as in the literature, the majority of rats demonstrate autotomy behaviors before the 4th post-operative week[21]. During this preliminary study, the rats were therefore closely followed for signs of autotomy during a 6–week period. This period was chosen as it should cover the critical time during which the rats may develop autotomy behaviors. If this preliminary study is unsuccessful, other options such as the use of repellant, restraining collars or anti-neuropathic medications (gabapentin, etc) will be evaluated. The adapted rat sciatic nerve model with acceptable autotomy will be used in the study of nerve regeneration in different nerve guidance tubes produced from human cultured cells.

3.3.3 *Evaluation of Hollow Tissue-Engineered Tubes and Hollow Tissue-Engineered Tubes Filled with Chitosan-Collagen Gel*

The in-vivo study of nerve regeneration through the previously designed tubes was repeated. For this purpose, the rat sciatic nerve model was used with 27 male rats of Lewis strain (Harlan Laboratories inc., Indianapolis, IN) between 250 and 300 grams. The sciatic nerve was transected and a nerve gap of 15mm was made. Four experimental groups were used: negative control group (n=6) consisting of nerve transection without repair; positive control group (n=6) consisting of an autograft; experimental lyophilized hollow nerve guide group (n=7); and experimental lyophilized hollow nerve guide filled with the collagen-chitosan gel group (n=8). The tubes used in the experimental groups for repair were prepared as described in section *3.1.4* and *3.1.5*.

3.3.4 *Evaluation of Lyophilized Filled Tissue-Engineered Tubes With and Without a Capillary-Like Network*

We chose to first evaluate the lyophilized filled tissue-engineered tubes, with and without a capillary-like network. For this study, the filled tubes were produced using the newly developed method. Fibroblast sheet were made with human fibroblast cells in the fifth passage. The fibroblast sheets were cultured for 30 days prior to rolling. For the endothelialized-filled tubes, HUVEC (fourth passage) were seeded onto the fibroblast sheet 5 days prior to rolling (day 25 of culture). The fibroblast sheets were rolled into filled tube with the technique developed in this thesis and described in section *3.1.6*. The filled tubes were matured in culture for 15 days and then lyophilized using a Gensis 12EL vacuum lyophilizer (Virtis, Gardiner, NY) for 40 hours. The filled lyophilized tubes were inserted into hollow nerve tubes produced with the protocol described in section *3.1.4*. Prior to surgery, the combined tubes were washed with PBS 1x supplemented with antibiotics (penicillin, gentamicin, and fungizone [amphotericine B]); they were kept in this solution at 4ºC until surgery.

For this study, 28 male rats of Lewis strain (Harlan Laboratories inc., Indianapolis, IN) between 250 and 300 grams were used. The sciatic nerve was transected and a nerve gap of 15mm was made. Five experimental groups were used: negative control group (n=4) consisting of nerve transection without repair; positive

control group (n=4) consisting of a simple transection and immediate repair; another positive control group (n=4) consisting of an autograft; experimental group consisting of a lyophilized filled nerve guide inserted into a hollow nerve guide (n=8); and a second experimental group consisting of the lyophilized filled nerve guide with endothelial cells inserted into a hollow nerve guide (n=8).

.

CHAPTER 4 - RESULTS

CHAPTER 4. RESULTS

4.1 Development of a Tissue-Engineering Approach to Produce Completely Biological Nerve Guidance Tubes from Cultured Human Cells

4.1.1 *Production of Filled Tissue-Engineered Nerve Tubes*

Previous attempts at rolling the fibroblast sheets without an inert tubular support produced tubes with an irregular, variable, and non-reproducible structure (Figure 4.1 and 4.2). Different techniques to roll the fibroblast sheets on themselves, without the use of a central tubular structure, were tried. These methods included using very fine straight and curved tweezers to roll or "fold" the fibroblast sheets on themselves, using a glass micro-pipette and a rubber tube as a tubular structure to "push" and force the fibroblast sheets to roll, and passing a fine suture into the fibroblast sheet to roll the sheet with the suture ends. All these different trials were unsuccessful in producing filled tubes reliably without damaging the fibroblast sheets.

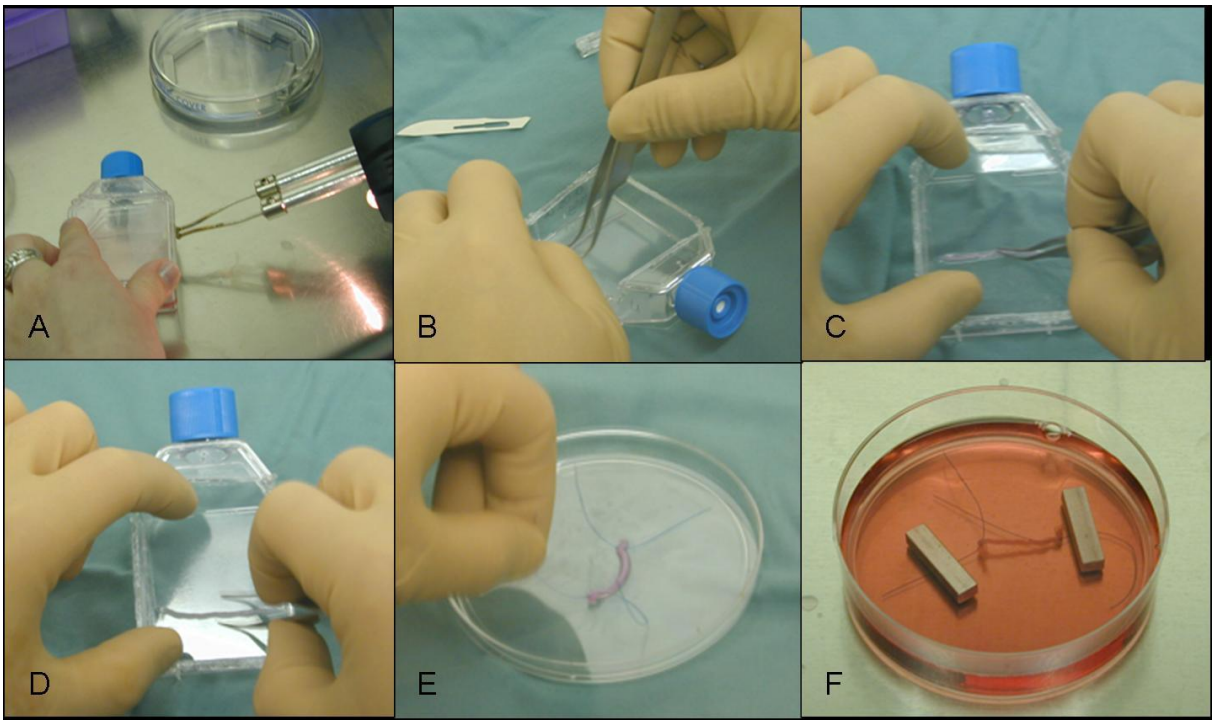

Figure 4.1 Previous Technique for Production of Filled Nerve Tubes

The fibroblast sheet culture flask was opened (A) and the sheet was detached (B). Using fine tweezers, the fibroblast sheet was rolled (C,D) into a filled tube (E) and matured under tension in a petri dish (F). Images from René Caissie, LOEX, with permission.

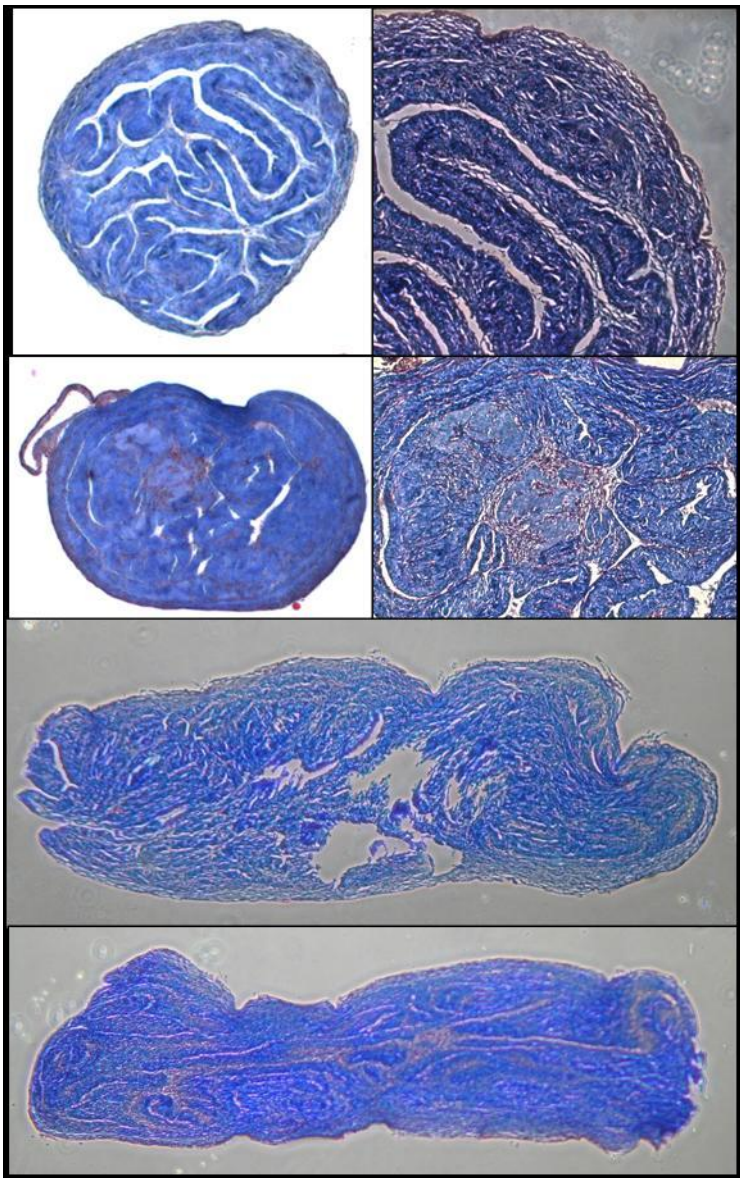

Figure 4.2 Structure of Filled Nerve Tubes from Previous Attempts

The irregular, variable, and non-reproducible structure of the previously produced filled nerve tubes is shown.

Finally, a method was found to be successful. The fibroblast sheet was detached of the culture flask. The edge (approximately 1mm) of the fibroblast sheet was folded on a thread using fine tweezers. The thread consisted of a simple sewing thread (Coats Koban, cotton wrapped polyester) composed of cotton and polyester (respectively 35% and 65%) that was sterilized in an autoclave before use. The edge was folded in the direction that the sheet was to be rolled. Each ends of the sewing thread was then rolled between two fingers. The fibroblast sheet followed the thread and rolled on itself. Both ends of the tubular structure made of fibroblast sheet were then closed with other segments of sewing threads and the central suture was removed. Occasionally, the central thread removal would create the tube to "telescope" out on the side the thread was removed (2/20 trials). With practice, the technique could be mastered and the removal of the central thread could be done without disturbing the tube except very rarely. The tubes could be put in traction in a petri dish using the sutures at each end to maintain an elongated tubular structure (Figure 4.3). For 24 hours after rolling, the tubes were covered with a polyvinyl alcohol (PVA) sponge (Merocel) secured with weights on each side of the tube to favor the adhesion of the outside fibroblast sheet layer. The tubes could be kept in culture for up to 30 days. This technique for rolling the fibroblast sheets on themselves was the one chosen for the production of the filled nerve tubes as it was the easiest and most reproducible method.

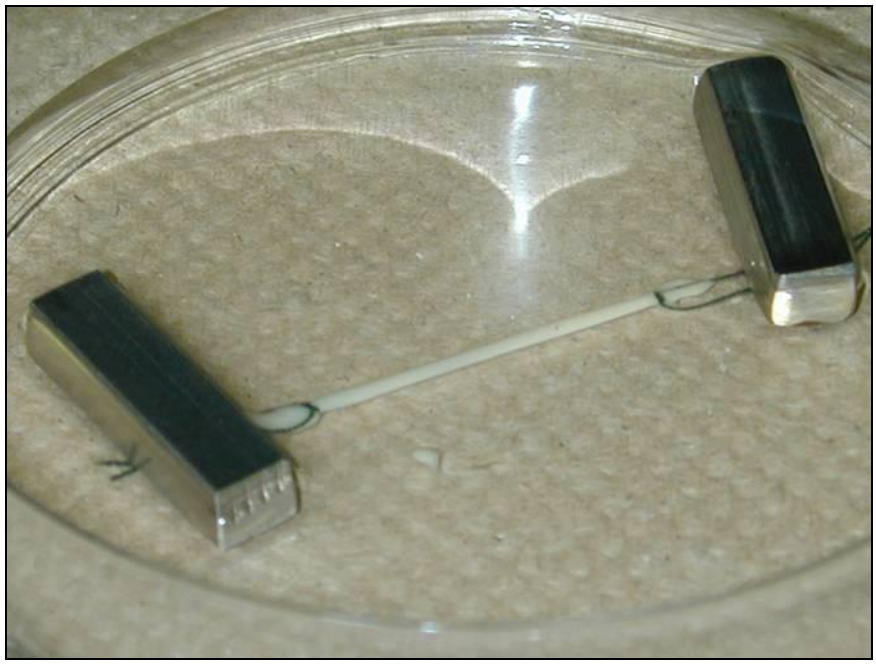

Figure 4.3 Filled Tissue-Engineered Nerve Tube

Filled tissue-engineered nerve tube is shown in traction in a petri dish

The filled tubes obtained by the newly developed technique had a reproducible architecture as evaluated qualitatively by histological appearance (Figure 4.4). The internal structure was composed of two distinct areas. First, the central core was composed of irregular layers of fibroblasts within their extracellular matrix. The central core was surrounded by 6 to 8 concentric layers of fibroblasts within their extracellular matrix. The tubes were approximately 1.8 mm in diameter; the concentric layers of fibroblast sheets were 20 to 50 µm thick and separated by 20 to 40 µm. The adherence of the external layer to the tube was initially variable, but as the time of maturation increases (14 and 31 days), the superficial layers were more adherent to the tubular structure. However, with maturation, the tubes became more compact.

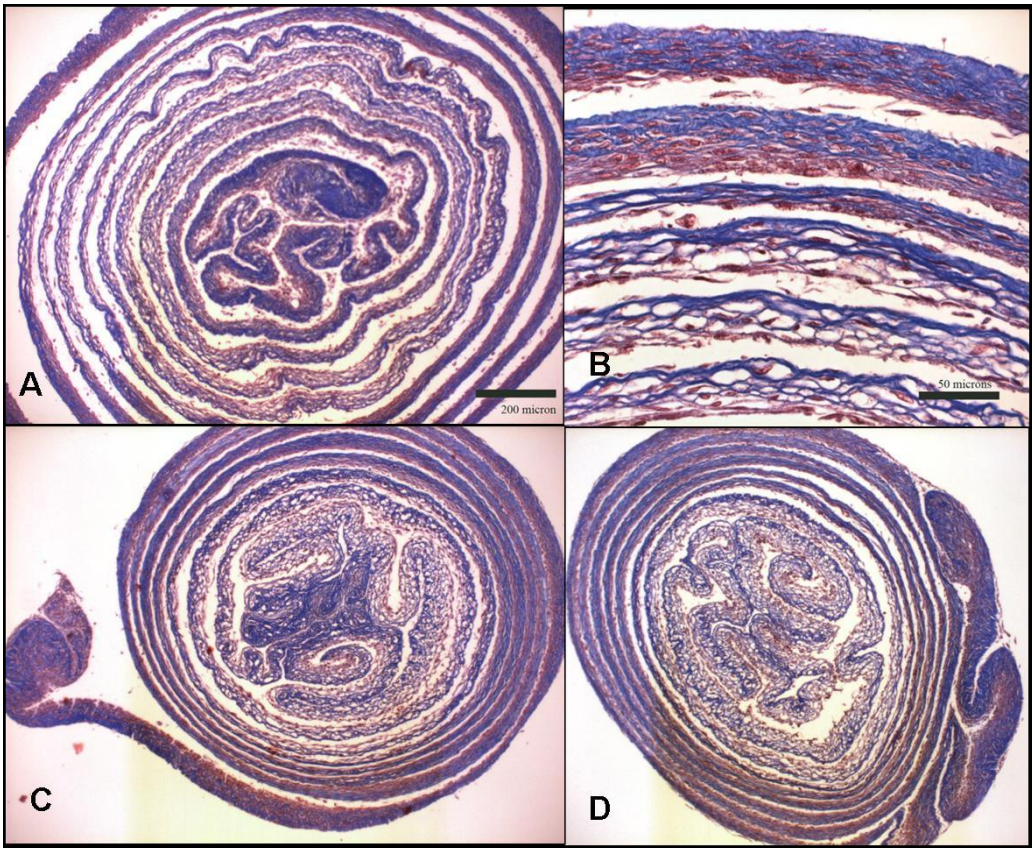

Figure 4.4 Filled Tissue-Engineered Nerve Tube

Histological sections with Masson's trichrome stain showing filled tissue-engineered nerve tubes rolled with the newly developed technique after 4 days of maturation. The irregular central core is surrounded by regular concentric layers of fibroblast within their extracellular matrix (A). Higher power view demonstrating the concentric layers of fibroblasts within their extracellular matrix (B). The external layer adherence to the tube is initially variable (C: easily detached, D: adherent). A 10X, B 40X, C 4X, D 4X.

As described above, the structure of the constructed tubes, especially when matured for longer periods to achieve adherence of the external layer, became compact. Attempts to modify the technique in order to make the tubes less compact and to increase the area available to the regenerating axons were made. The fibroblast sheets were rolled with the central sewing thread as described above. In addition, 1 to 5 additional threads were included between the fibroblast layers during the rolling. The threads were kept in the tubes for 7 days, after which they were removed, which damaged one third (3/9) of the tubes. When examined by histology, the structure of the tubes was more compact than the tubes rolled without extra threads. This technique was not pursued.

The constructed filled tubes could be sutured with microsurgical technique under loop or microscopic magnification, but with difficulty. To give an external layer allowing a resistance to traction permitting micro-suturing, the developed guidance channels were combined: the hollow tubes previously designed (as described in the material and methods section) were combined with the filled tubes to produce a "combined tube". The physical properties of the hollow tubes allowed micro-suturing. These "combined tubes" could be formed with an internal living filled tube, although the living filled tubes were very fragile to manipulation. The liophylized filled tube could be inserted within the lyophilized hollow tube without difficulty as its diameter was significantly reduced, providing the external support necessary for suturing (Figures 4.5 and 4.6).

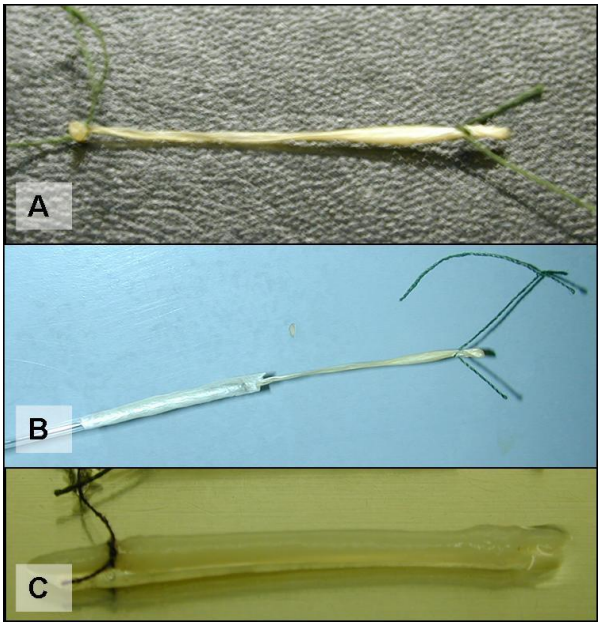

Figure 4.5 Lyophilized Filled Nerve Tube Combined with Hollow Nerve Tube
The lyophilized filled tissue-engineered nerve tube (A) is inserted into a lyophilized hollow nerve tube (B). This technique is relatively easy as the diameter of the hollow tube is slightly larger than the diameter of the filled tube. The combined nerve tube is then rehydrated (C). The hollow nerve tube has sufficient resistance to sustain microsuturing.

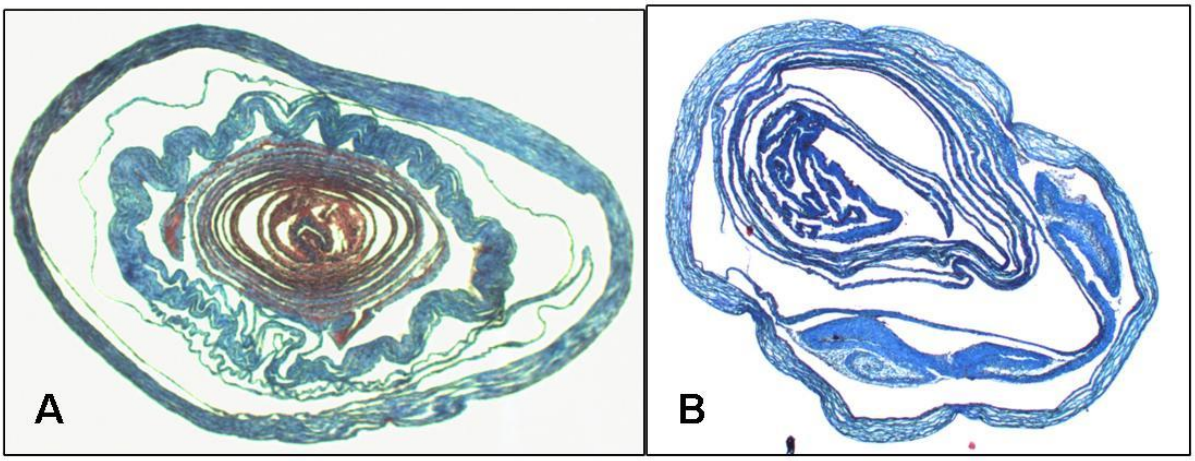

Figure 4.6 Lyophilized Filled Nerve Tubes Combined with Hollow Nerve Tubes
Histologic section stained with Masson's trichrome demonstrating the structure of the combined nerve tubes. The filled "internal" tissue-engineered nerve tube can be alive (A) or lyophilized (B). 4X

4.1.2 *Production of Rat Fibroblast Sheets*

The rat fibroblasts cultured in an attempt to produce fibroblast sheets, as described in the material and methods, was unsuccessful. The three rat fibroblast cell lines did not produce significant extracellular matrix when cultured in the medium used for human fibroblast (DME, supplemented with fetal calf serum, antibiotics, and sodium ascorbate). At 29, 35, and 43 days of culture, the rat fibroblast sheets were very thin. At 43 days, the sheets could be detached from the flask, but they could not sustain any manipulation.

The culture medium was modified, adding glutamine, as it was shown by other investigators in the LOEX to increase extracellular matrix formation in porcine fibroblasts and some reports in the literature report supplementation of the culture medium of rat fibroblasts with glutamine[68]. Glutamine was added to the fibroblast culture medium at different concentration (0mM, 5mM, 7.5mM, and 10mM), the number of rat fibroblast seeded into the culture flask was varied (0.3 million cells/25cm^2 flask, as initially studied, and 0.6 million cells/25cm^2 flasks), and fibroblasts of an earlier passage (2nd) were used. Three samples were made for each condition. Despite 36 days of culture, there was no significant production of extracellular matrix allowing manipulation of the sheets.

Because of initial trials of freshly extracted fibroblast by other LOEX investigators produced some rat fibroblast sheets, it was hypothesized that the freezing process of the rat fibroblasts cells might have changed their potential to form extracellular matrix. Moreover, skin from different areas and rat fibroblasts of Lewis strain (chosen strain to test regeneration) could have different potentials to form extracellular matrix. Fibroblasts from the skin of Lewis rats (skin from the back (5 separate), ears (8 pooled), and abdomen (2 pooled)) were extracted according to the protocol described in the material and methods. The fibroblasts were seeded at the second passage (0.3 million cells/ 25 cm^2 culture flask) for the production of fibroblast sheets. Glutamine was added to the fibroblast culture medium at different concentration (0mM, 5mM, 7.5mM, and 10mM). At 35 days of maturation, the fibroblast sheets were too thin to be manipulated. After 54 days of culture, the extracellular matrix was more important and some fibroblast sheets could be manipulated onto a paper frame. At 70

days of culture, all cultures (10 different conditions) had formed sufficient extracellular matrix to allow manipulation of the fibroblast sheet into a paper frame. The best fibroblast sheets seemed to be when the fibroblasts were cultured into medium supplemented with 10mM of glutamine.

4.2 Production of Nerve Guidance Tubes with a Capillary-Like Network from Cultured Human Cells

4.2.1 *Formation of a Capillary-Like Network*

HUVEC seeded on fibroblast sheets have the ability to create a capillary-like network within the newly designed tissue-engineered nerve guides described above. On histologic section, capillary-like structures were mainly observed at 14 and 31 days of maturation (Figure 4.7). These capillary-like structures could be seen within the different layers of the tubes. The formation of capillary-like structure formed by endothelial cells was confirmed by immunofluorescence studies using anti-PECAM-1 antibodies; only a few cells were staining positive after 4 days, but capillary-like structures staining positive were seen after 14 days (Figure 4.8). The capillary-like formation was confirmed by co-localization of laminin staining with PECAM-1 on immunofluorescence studies (Figure 4.8).

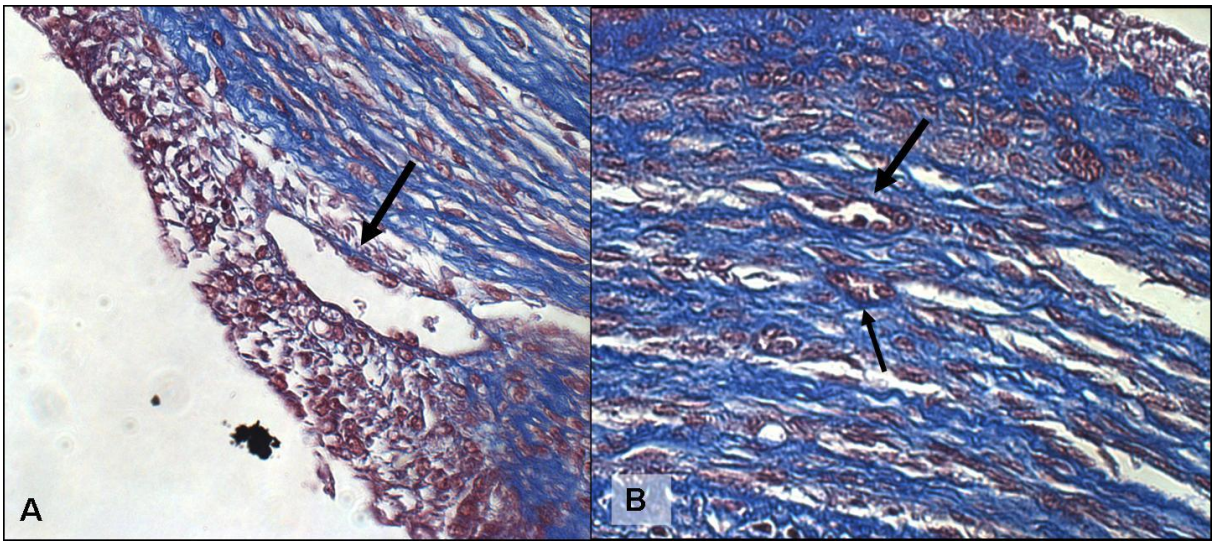

Figure 4.7 Capillary-Like Structure within Filled Tissue-Engineered Nerve Tubes
Histologic section stained with Masson's trichrome showing capillary-like formations (arrows) within the concentric layers of fibroblast within their extracellular matrix. The endothelial cells (HUVEC) were seeded prior to rolling and the filled tubes were kept in culture for 14 (A) and 16 (B) days. Images taken at 40X.

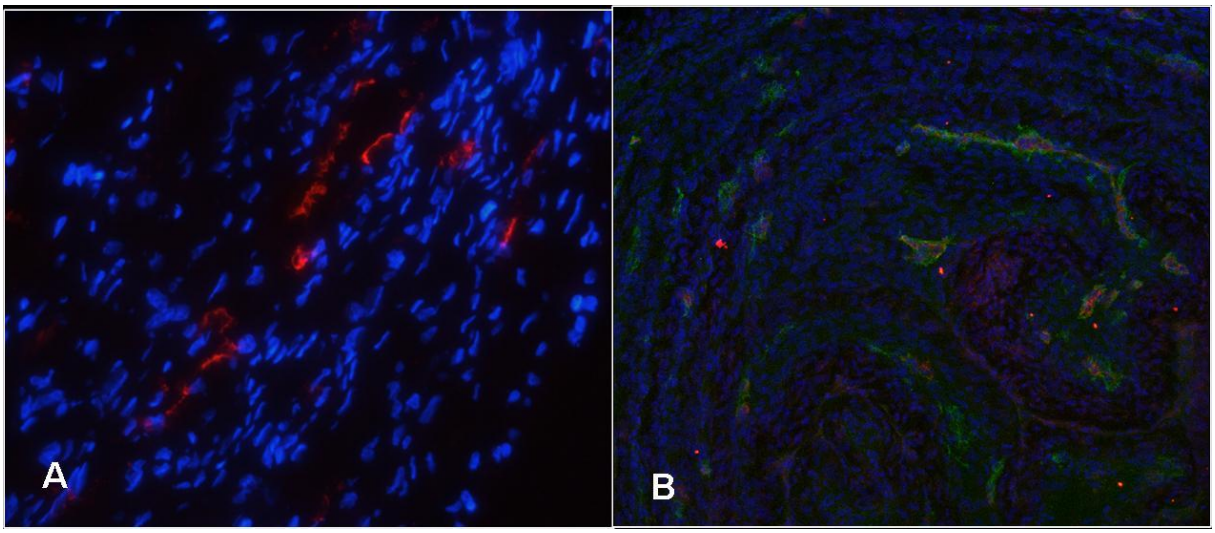

Figure 4.8 Capillary-Like Structure within Filled Tissue-Engineered Nerve Tubes

Immunofluorescence studies confirm the presence of capillary-like structure within the filled tissue-engineered nerve tubes. These tubes were matured for 1 (B) and 14 (A) days after rolling. The capillary-like structures, formed by endothelial cell, are revealed by anti-PECAM-1 antibodies (A). The nuclei are stained with Hoescht. There is colocalization of the anti-PECAM-1 antibody and anti-laminin antibody, confirming the presence of capillary-like structures (B). A 40X, B 10X.

4.2.2 *Characterization of the Growth and Formation of a Capillary-Like Network*

The use of HUVEC-GFP was very helpful in better characterizing their behavior and potential to form capillary-like networks within the tissue-engineered nerve tubes (Table 4.1). As maturation time in the rolled fibroblast sheet increases (experiment 1), the HUVEC-GFP cells tend to elongate and start forming connection between the cells (Figure 4.9). If the HUVEC-GFP are cultured on the fibroblast sheets before rolling them into tubes, they form extensive capillary-like networks. Both the initial seeding concentration of the HUVEC-GFP on the fibroblast sheet and the maturation time have an effect on the capillary-like network formation (experiment 2). Lower initial HUVEC-GFP seeding concentrations (0.3 and 0.6 million of cells/ 25 cm^2) promotes the formation of capillary-like networks, whereas higher initial HUVEC-GFP seeding concentrations produces sheets of confluent endothelial cells with less capillary-like network formation (Figure 4.10). Maturation time also influences the formation of capillary-like networks; these start to form around 9 days post seeding, and increases with time, up to 35 days post-seeding (Figure 4.11). When rolled into filled tubes, the HUVEC-GFP and some capillary-like connections persist (Experiment 2; Figure 4.12). When mid-range (0.6 to 0.9 million cells /25cm^2) initial seeding concentrations are used (experiments 4 and 5), the capillary-like network obtained within the tubes was thick and had a "chicken-wire appearance" which remains broad with maturation (Figure 4.13).

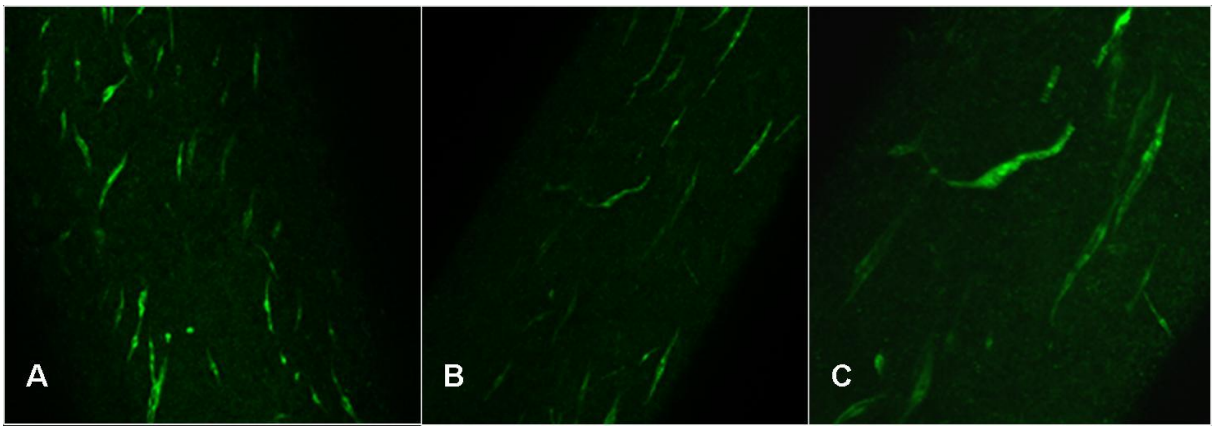

Figure 4.9 HUVEC within Filled Tissue-Engineered Nerve Tubes
Confocal images of living filled tissue-engineered nerve tubes seeded with HUVEC-GFP. As maturation time in the rolled fibroblast sheet increases (A= 16 days; B, C= 30 days), the HUVEC-GFP cells tend to elongate (B) and start to form connections (C).
A 10X, B 10X, C 20 X

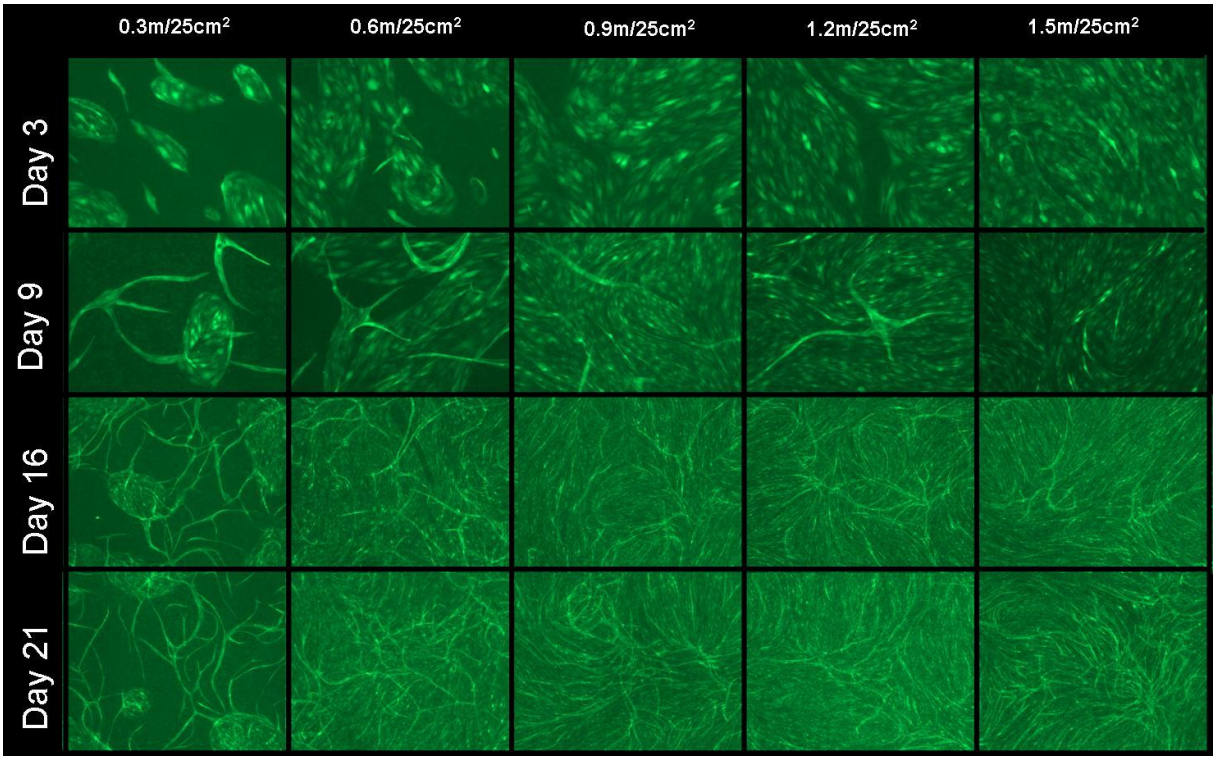

Figure 4.10 Capillary-Like Network Formation on Fibroblast Sheets

The capillary-like network formation after seeding of HUVEC on fibroblast sheets is influenced by the initial seeding concentration and by the maturation time. Capillary-like structures appear around the 9th day after seeding. A lower initial seeding concentration seems to favor capillary-like structure formation whereas a higher initial seeding concentration favors the formation of confluent sheets of endothelial cells.

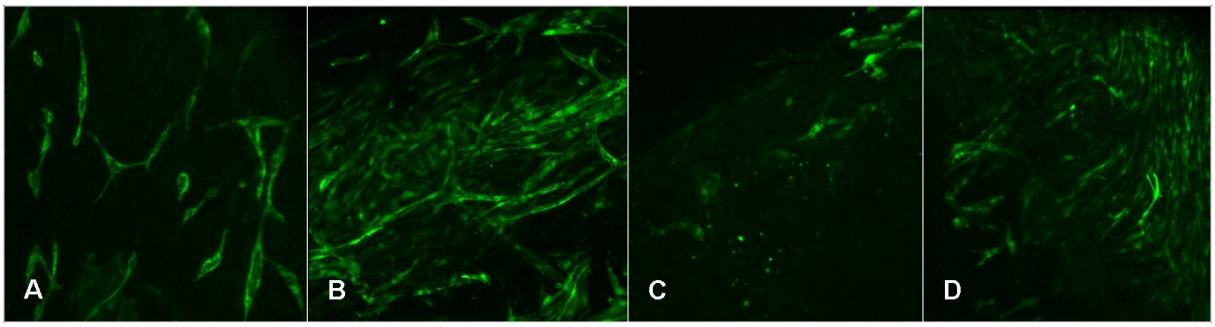

Figure 4.11 Capillary-Like Network within Filled Tissue-Engineered Tubes

Confoncal images show the survival of the HUVEC-GFP and the capillary-like network formation within filled tissue-engineered tubes after 7 days of maturation. The appearance of the HUVEC-GFP cells and capillary-like network formation varies depending of the initial HUVEC-GFP seeding concentrations (A=0.3 million cells/25cm^2; B= 0.6 million cells/25cm^2; C=1.2 million cells/25cm^2; D=1.5 million cells/25cm^2).

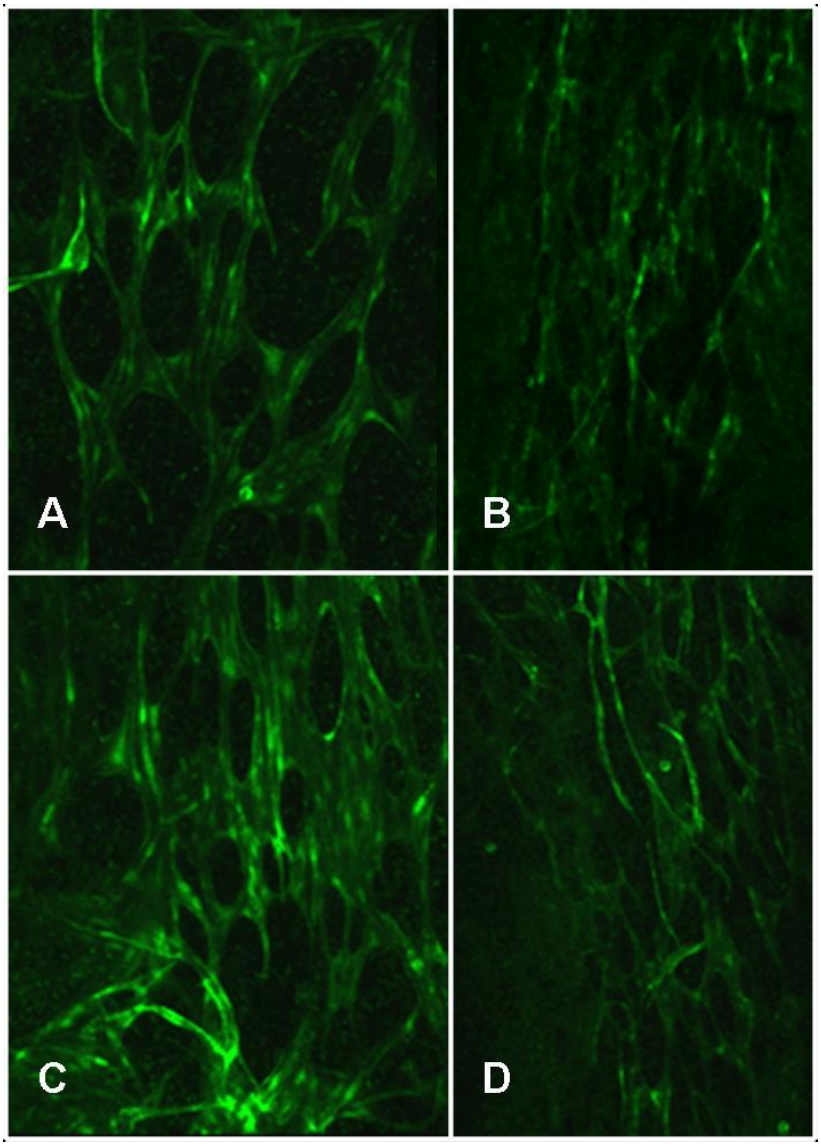

Figure 4.12 Capillary-Like Network within Filled Tissue-Engineered Tubes

When mid-range initial HUVEC-GFP seeding concentrations (0.6 (A, B) to 0.9 (C, D) million cells /25cm^2) are used, the capillary-like network obtained has a "chicken-wire appearance". The capillary-like network remains broad with maturation (15 days (A, C) and 30 days (B, D) of maturation are shown). A 20X, B 10X, C 20X, D10 X.

4.2.3 *Exploration of a Different Method of Fibroblast Sheet Assembly for Optimal Formation of Capillary-Like Network*

In an attempt to optimize the formation of the capillary-like network, a different method of assembling the fibroblast sheets before forming the filled tubes was designed. This new method of assembly, the "sandwich technique", consisted of assembling two sheets of fibroblast seeded with endothelial cells together and culture them together prior to the formation of the filled tubes (Figure 4.13). The fibroblast sheets were first seeded with HUVEC-GFP and matured for a week prior to assembly. Each HUVEC-GFP seeded fibroblast sheet was then delicately detached from the culture flask, placed on sterilized (autoclaved) acetate, and combined with another sheet, each with the endothelial-seeded surface facing the other. The acetates were removed and the combined sheet was placed in a petri dish and kept under tension to prevent contraction. A PVA sponge (Merocel) secured with weights was placed on the assembled sheets for 24 hours to increase adhesion between the two sheets. These assembled sheets were kept in culture and then rolled into tubes (technique developed and described in this thesis).

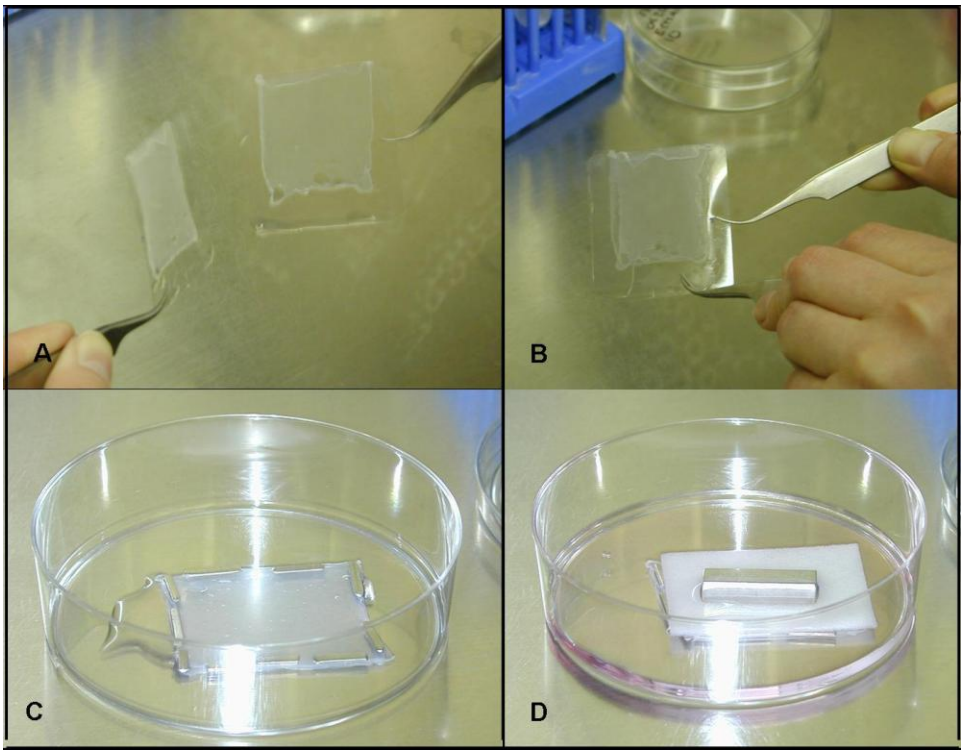

Figure 4.13 Technique for the Assembly of Endothelialized Fibroblast Sheets
Each HUVEC-GFP seeded fibroblast sheet is placed on an acetate (A), and combined with another sheet, each with the endothelial-seeded surface facing the other (B). The acetates were removed and the combined sheet was placed in a petri dish and kept under tension to prevent contraction (C). A Merocel is placed on the assembled sheets for 24 hours to increase adhesion between the two sheets (D).

The tubes formed with this modified technique had a regular structure, but were more compact (Figure 4.14). Formation of multiple capillary-like structures was observed (Table 4.1), both during the maturation period (Figure 4.15) and after rolling (Figure 4.16). With an increased initial HUVEC-GFP seeding concentration (0.9million cells/25cm^2), the capillary-like network had a wide "chicken-wire" appearance. These capillary-like networks persisted after rolling the assembled sheets. Their appearance was almost sheet-like with higher initial concentrations. However, it was possible to obtain a longitudinal capillary-like network resembling an intrinsic vascular network found in nerves (at 0.3million cells/25cm^2 seeding concentration) (Figure 4.17).

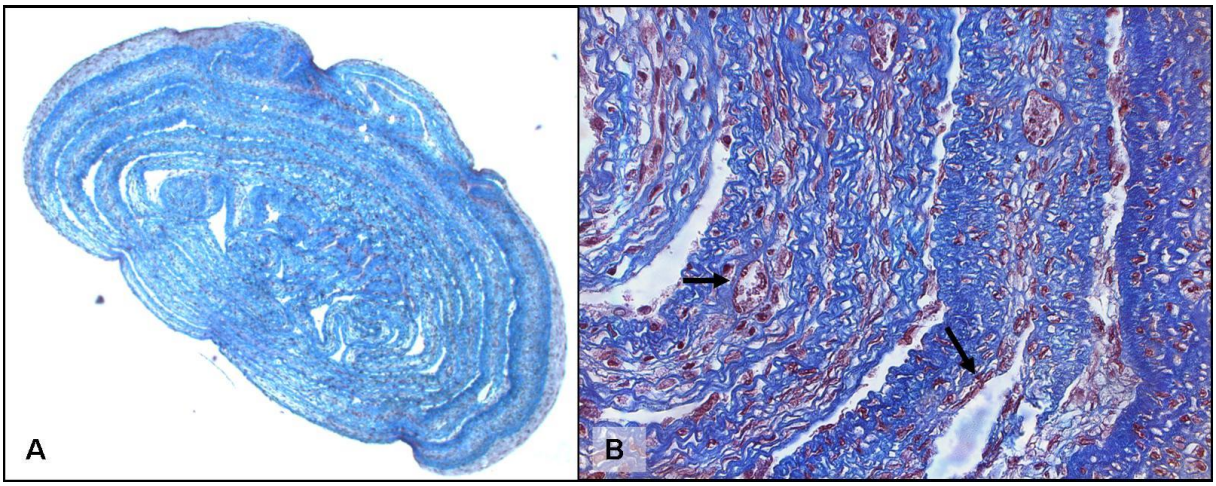

Figure 4.14 Filled Tissue-Engineered Nerve Tubes with Sheets Assembly

Filled tissue-engineered nerve tube formed after assembly of two endothelialized fibroblast sheets. This tube was matured in culture for 30 days. The structure is regular but more compact than the nerve tubes formed with a single fibroblast sheet (A). Multiple capillary-like structures (arrows) are seen within the concentric layers of the tube (B).

Masson's trichrome; A 4X, B 40X.

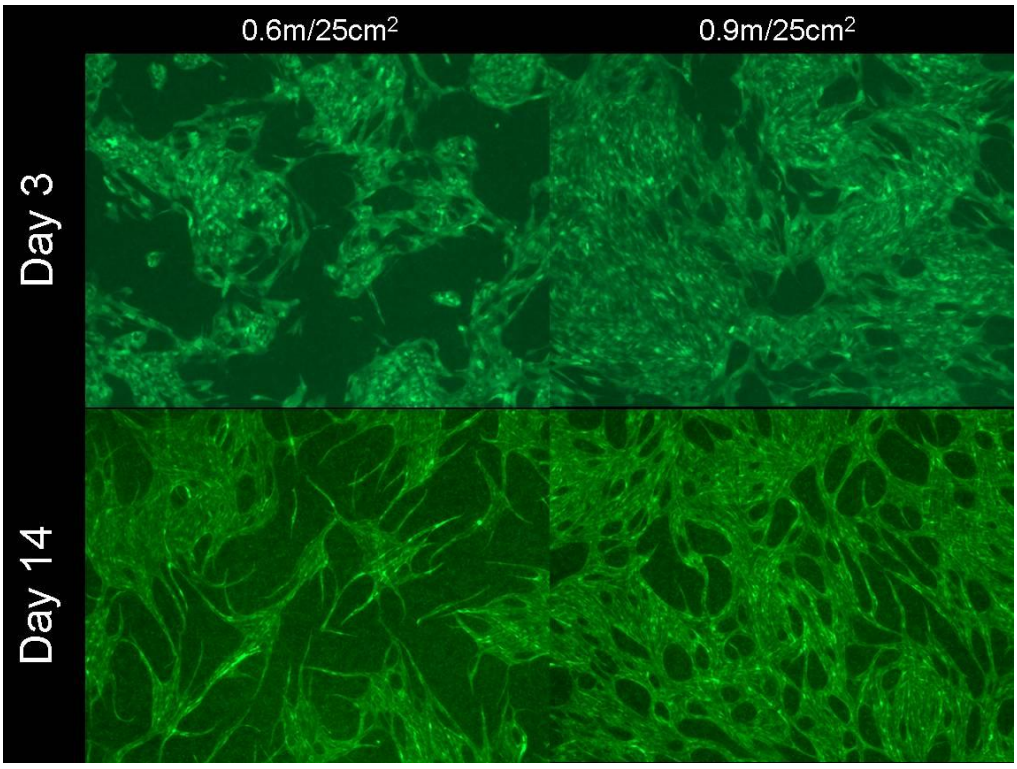

Figure 4.15 HUVEC-GFP Capillary-Like Network in Assembled Fibroblast Sheets

The HUVEC-GFP form capillary-like networks which are wider when higher initial seeding concentration are used.

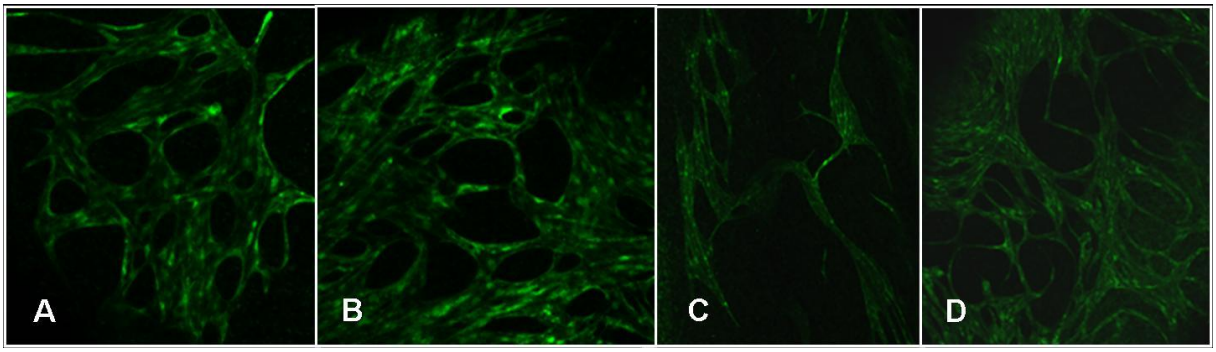

Figure 4.16 Capillary-Like Network in Filled Tubes Formed with Assembled Fibroblast Sheets

The capillary-like network in the filled tubes formed with assembled fibroblast sheets are wide and resemble a "chicken-wire" appearance. These wide capillary-like structures are present early after the tube formation (day 4 in A and B) and persist with maturation (day 30 in C and D). This phenomenon occurs at mid-range initial HUVEC-GFP seeding concentrations (0.6 million cells /25cm^2 in A and C; 0.9 million cells/25cm^2 in B and D). A 20X, B 20X, C 10X, D 10X.

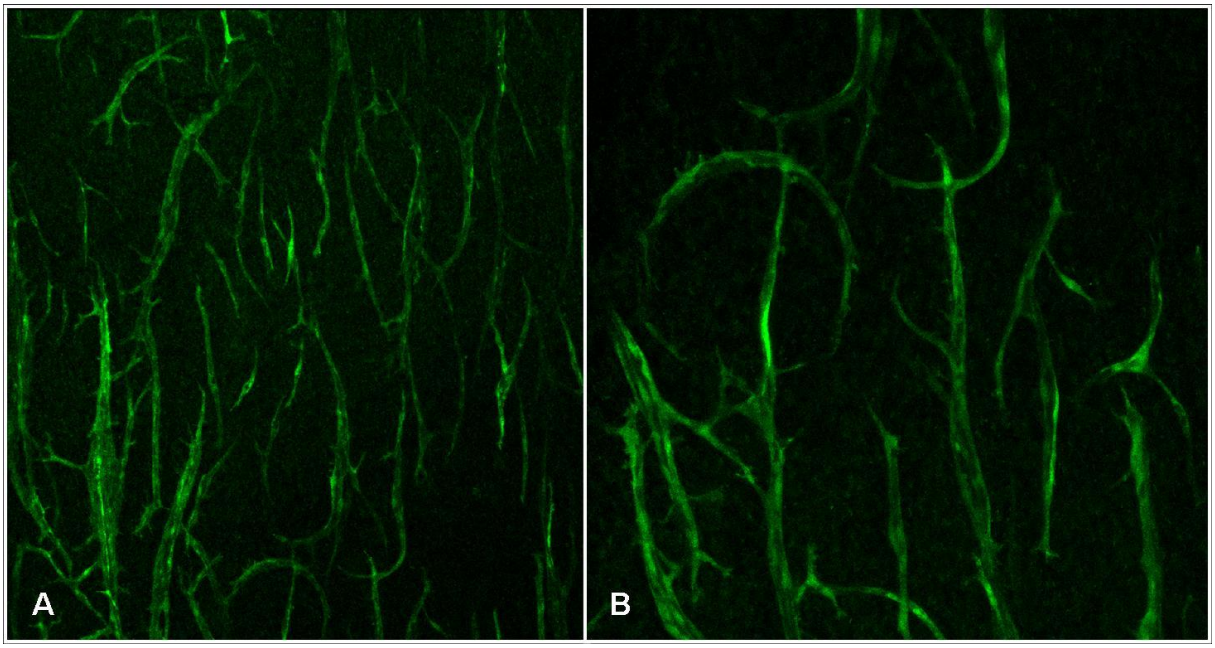

Figure 4.17 Longitudinal Capillary-Like Network within Filled Tissue-Engineered Nerve Tubes

Longitudinal capillary-like network resembling native neural vasculature was obtained by rolling assembled fibroblast sheets into nerve tubes, after 30 days of maturation. Initial HUVEC-GFP seeding concentration was 0.3 million cells/25cm^2). A 10X, B 20X.

Table 4.1 Capillary-Like Network Formation using HUVEC-GFP

Experiments	Fibroblast sheet maturation (days)	HUVEC-GFP seeded (million per 25cm^2)	Maturation HUVEC-GFP on fibroblast sheet (days) prior to…		Maturation of construct (days) after…		Capillary-like network formation
			Assembly	Rolling	Assembly before rolling	Rolling	
1	26	0.3		5		1	Cells only
						4	Cells only
						16	Cells only
						30	Elongated cells
2	25	0.3		21		8	Few networks
		0.6				8	Few networks
		0.9					
		1.2				8	Cells only
		1.5				8	Cells only
3	28	0.6		44		8	Few networks
4	26	0.6		22		1	Network
						4	Wide network
						15	Wide network
						30	Important network
5	26	0.9		22		1	Cells only
						4	Few wide networks
						15	Wide network
						30	Nice network(2/3)
6	28	0.3	7	16	9	5	Cells only
						15	Some branching
						30	Important network
7	26	0.6	7	22	15	1	Cell sheet
						4	Wide network
						15	Wide network
						30	Few Networks
8	26	0.9	7	22	15	1	Cell sheet
						4	Wide network
						15	Wide network
						30	Wide network

4.3 Study of nerve regeneration in different nerve guidance tubes produced from human cultured cells.

4.3.1 *Adaptation of the Rat Sciatic Nerve Model to Reduce Autotomy*

This preliminary study with Lewis rats was successful. All 6 rats were kept alive, without signs of autotomy for the duration of the experiment (6-week) despite sciatic-innervated muscle denervation (Figure 4.18). The change of the rat strain, from Sprague-Dawley to Lewis rats, was sufficient to have a rat sciatic nerve model with acceptable autotomy rate (0 in 6 rats).

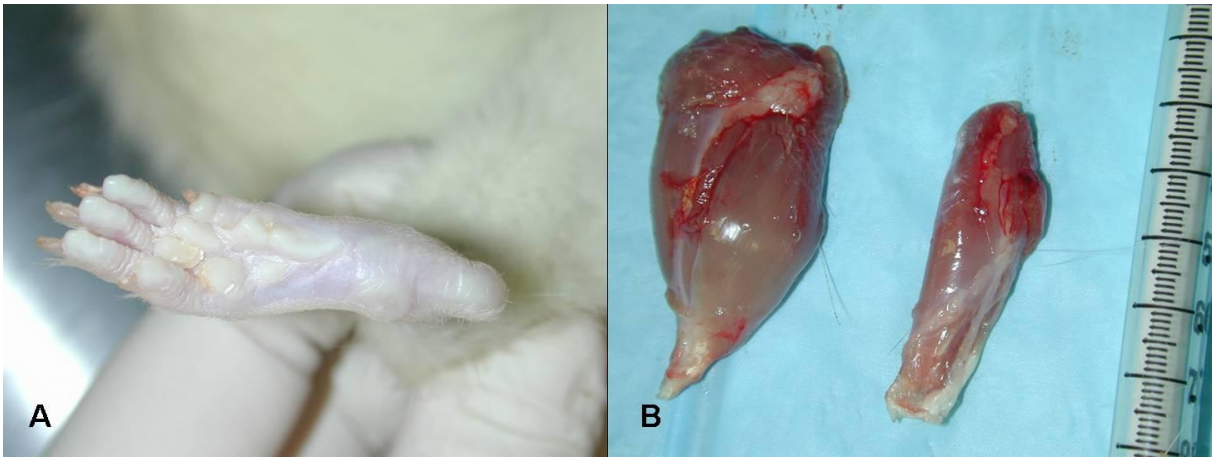

Figure 4.18 Results of Preliminary Study with Lewis Rats
None of the rats had signs of autotomy during the study follow-up. The hindpaw showed decreased toe spread but no sign of autotomy (A). Adequate muscle denervation was achieved as shown (B) by the difference in the gastrocnemius muscle size (normal left, denervated right).

4.3.2 *Evaluation of Hollow Tissue-Engineered Tubes and Hollow Tissue-Engineered Tubes Filled with Chitosan-Collagen Gel*

The in-vivo study of nerve regeneration through the previously designed tubes, lyophilized hollow nerve guides and the lyophilized hollow nerve guides filled with the chitosan-collagen gel was overall negative. Both experimental tubes did not support nerve regeneration across a 15mm nerve gap; the details are given below.

All 27 rats survived the surgery, post-operative and follow-up period. Clinically, despite manual daily physiotherapy to the experimental leg, many rats developed contractures. This phenomenon was most common for the rats of the positive control (autograft) group (1 of 6 rats at 8 weeks, 4 of 6 rats at 12 weeks, and all 6 rats at 15 weeks). Because of this, the motor functional assessment, i.e. walking track analysis and measurement of the sciatic function index, was limited (Figure 4.19). We could still demonstrate that over time (between 3 weeks post-operatively and 15 weeks post-operatively), there was a change of the SFI (mean difference 9.81±2.85, p=0.0003) when all groups were considered. However, at the completion of the study (15 weeks), there was no difference between the SFI of the different experimental groups (p=0.51).

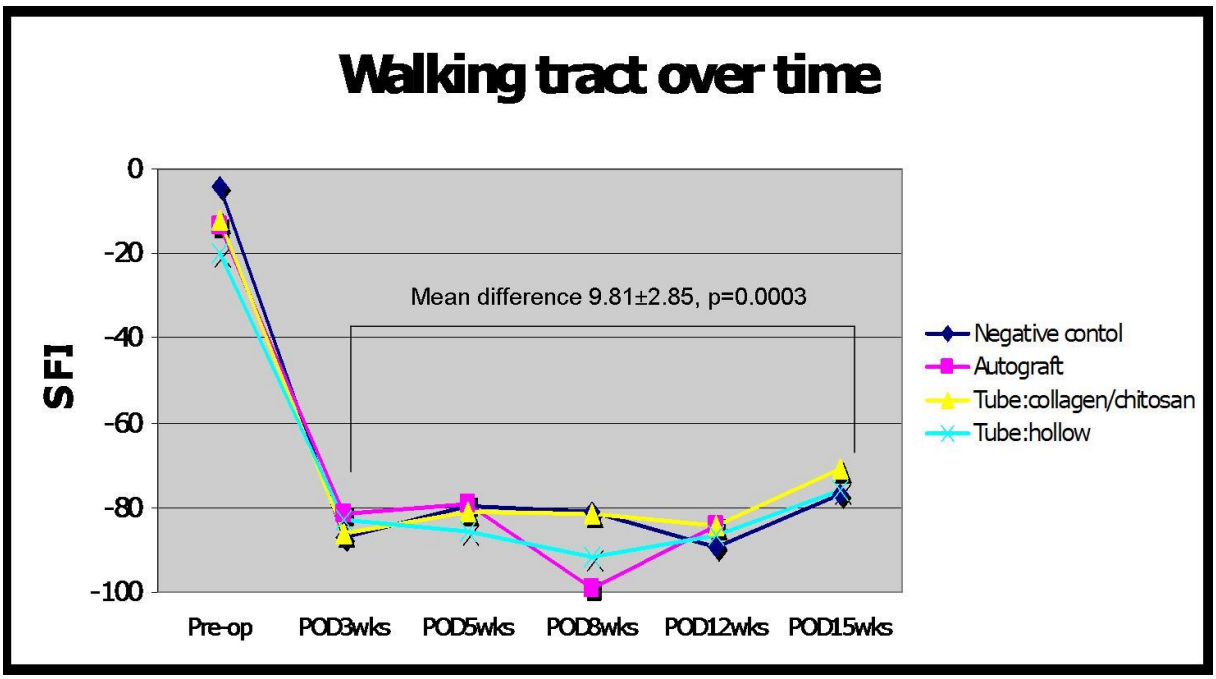

Figure 4.19 Sciatic Function Index over Time in the Different Experimental Groups

The sensory functional recovery is difficult to interpret using the results obtained by the Neurometer (Figure 4.20). In all groups, the experimental hindpaw was clinically sensitive to manipulation. The percent change between the intensity at which the rat was responsive from the experimental hindpaw compared to the normal contralateral hindpaw was calculated without difficulty. The intensity at which the animal demonstrated discomfort from each frequency could be reliably reproduced, i.e. there was minimal variation in the intensity threshold at a given post-operative time and given frequency for a particular animal. However, important variations were present between individual animals, even of the same group, at a given time. In some animals, the threshold intensity was smaller in the experimental hindpaw compared to the contralateral hindpaw, even as early as 3 weeks post-operatively (i.e. they required less intensity to react to the stimulus in the "denervated insensate" hindpaw). Other animals within the same experimental group required, as expected, higher threshold intensity to react to the stimulus applied. No consistent statistically significant differences were found between the experimental groups at a given time post-operatively and at a given frequency.

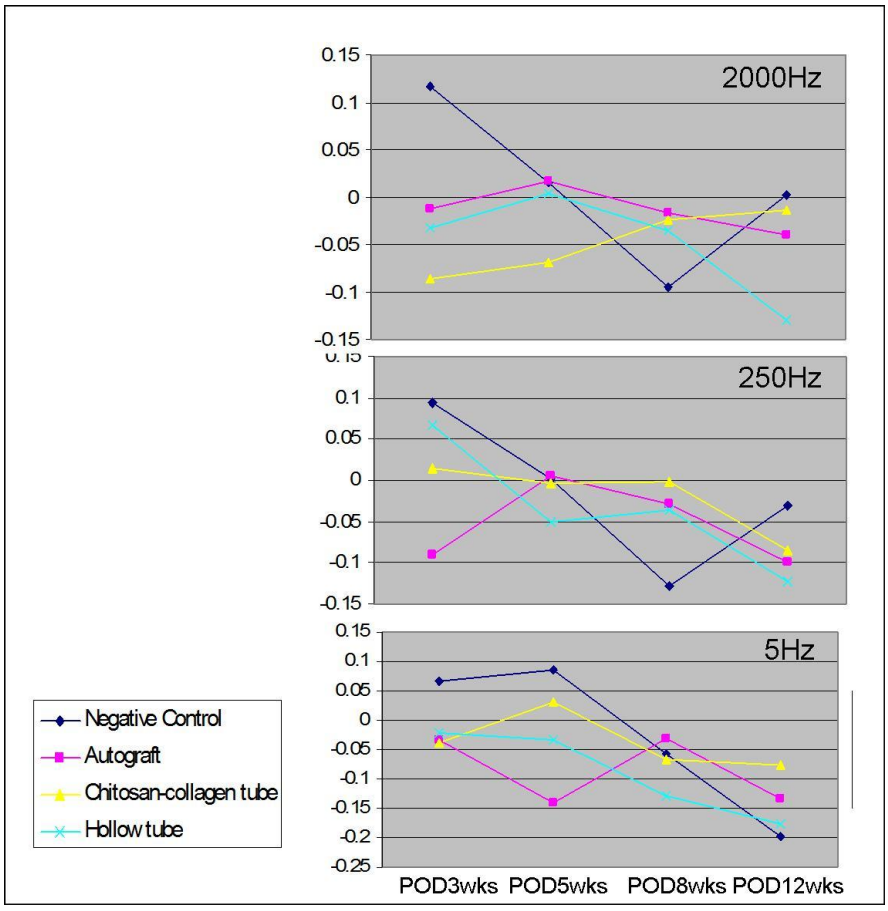

Figure 4.20 Neurometer Testing in the Various Experimental Groups over Time
(Results shown as % change of sensitivity threshold from contralateral normal extremity)

When the surgical sites were reopened at the completion of the study period, a discolored collection of material was found around the edges of the reconstructed tubes in certain rats. These collections resembled abscess formation, but no culture was taken. They were present in half (4/8) of the hollow tube filled with the chitosan-collagen gel, but in none of the other groups.

The total number of myelinated axons present at three levels (i.e., proximal to the graft, in the graft, and distal to the graft) was evaluated for the autografts, the hollow nerve tubes, and the hollow tubes filled with the chitosan-collagen gel (Figure 4.21). Because of restrictions in financial resources and of the absence of positive results in the experimental groups, the number of myelinated axons was not assessed in the negative control group. However, no significant difference would be expected. The autograft group served as our positive control and we did not assess the number of myelinated axons present in a normal rat sciatic nerve. The data available in the literature (myelinated axons estimated to be 8230 ± 220 in the rat sciatic nerve)[135] cannot be compared to our results as our assessment included only a portion of the nerve. There was no statistically significant difference between the number of myelinated axons present proximal to the graft between the different groups ($p=0.18$). However, there were significantly more myelinated axons present within the autografts (1862.67±311.47) than within the hollow nerve tube (368.00±288.36, $p=0.0066$) or the hollow tube filled with the chitosan-collagen gel (487.75±269.74, $p=0.0098$). There was also more myelinated axons present distal to the graft in the autograft group (1129.17±248.02) than within the hollow nerve tube (48.29±229.62, $p=0.0132$) or the hollow tube filled with the chitosan-collagen gel (163.88±214.79, $p=0.0226$).

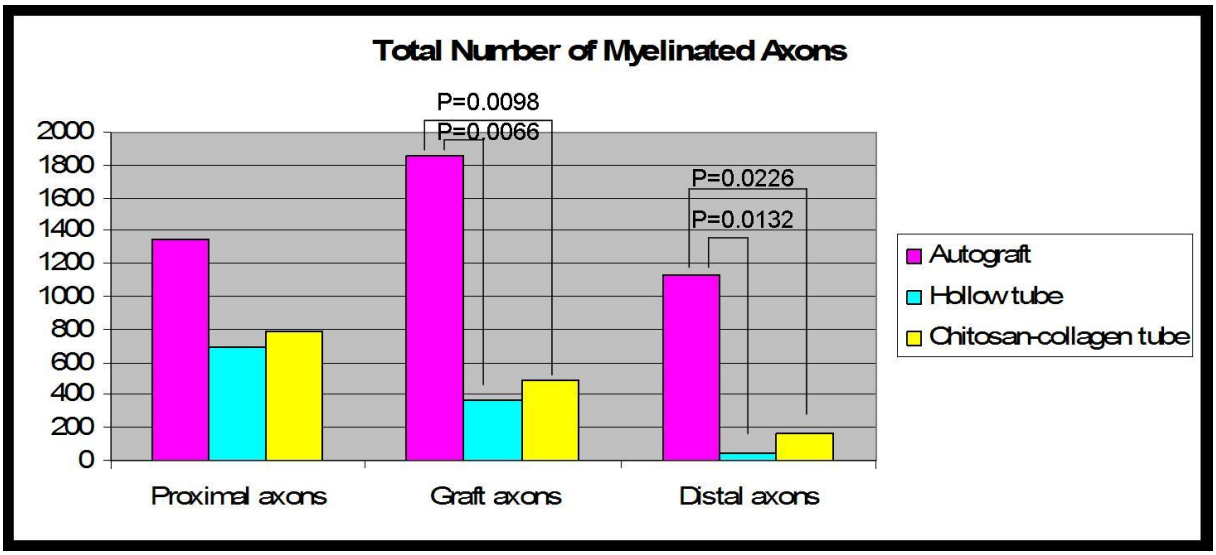

Figure 4.21 Total Number of Myelinated Axons in the Different Experimental Groups

The ratio of the experimental versus control gastrocnemius weight (Figure 4.22) was also significantly different (p=<0.0001) between the autograft group (0.54±0.03) and the nerve resection group (0.15±0.03), the hollow nerve tube group (0.15±0.03), and the hollow tube filled with the chitosan-collagen gel group (0.18±0.03).

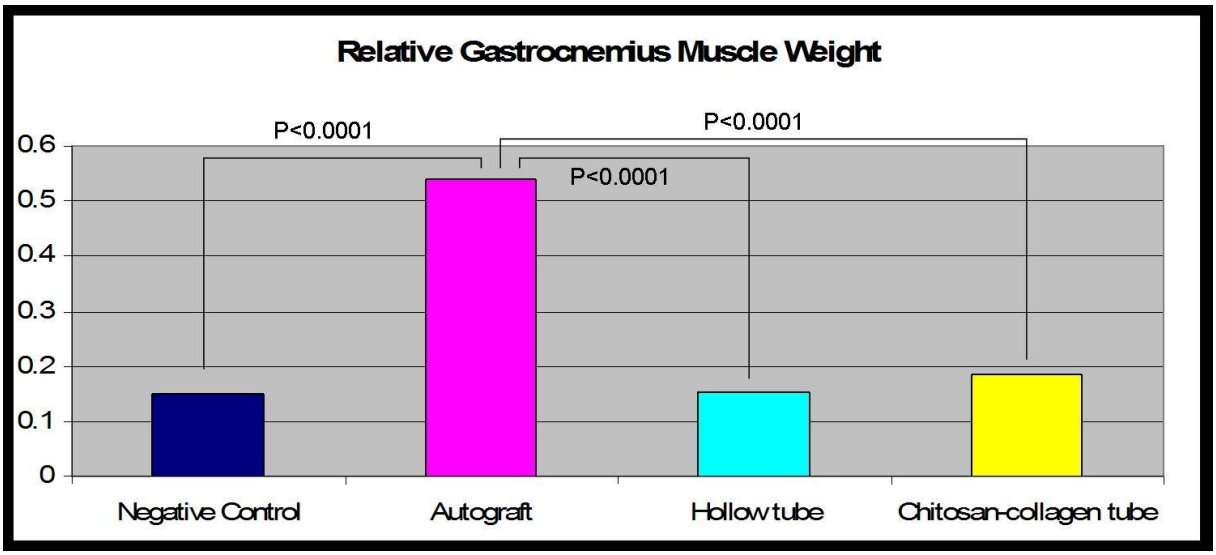

Figure 4.22 Relative Gastrocnemius Muscle Weight

4.3.3 *Evaluation of Lyophilized Filled Tissue-Engineered Tubes With and Without a Capillary-Like Network*

The in-vivo study of nerve regeneration through the lyophilized filled tissue-engineered tubes with and without a capillary-like network did not demonstrate significant axonal regeneration.

The quality of the reconstructed "combined" nerve tubes at surgery was adequate and the repair was easier to achieve. All 28 rats survived the surgery, post-operative and follow-up period. Clinically, despite manual daily physiotherapy to the experimental leg, many rats again developed contractures. As for the previous studies, the experimental hindpaw was sensitive to manipulation in all experimental groups.

The motor functional assessment, i.e. walking track analysis and measurement of the sciatic function index, was again limited because of the development of contractures in some rats. As for the previous experiments, this phenomenon was most common for the rats of the autograft and direct repair groups (contractures preventing walking tract analysis in 3 of the 8 rats at 6 weeks, 5 of 8 rats at 9 and 15 weeks, and 6 of 8 rats at 12 weeks). It was still possible to demonstrate (Figure 4.23) that at 6 and 9 weeks, a significant improvement in the SFI of the direct repair group was present compared to the transaction and tissue-engineered nerve tubes groups ($p<0.005$). At 9 weeks, the SFI of the direct repair group was also significantly better than the autograft group ($p=0.0002$). At completion of the study (15 weeks), the SFI was significantly better in the direct repair group compared to all other experimental groups ($p<0.007$).

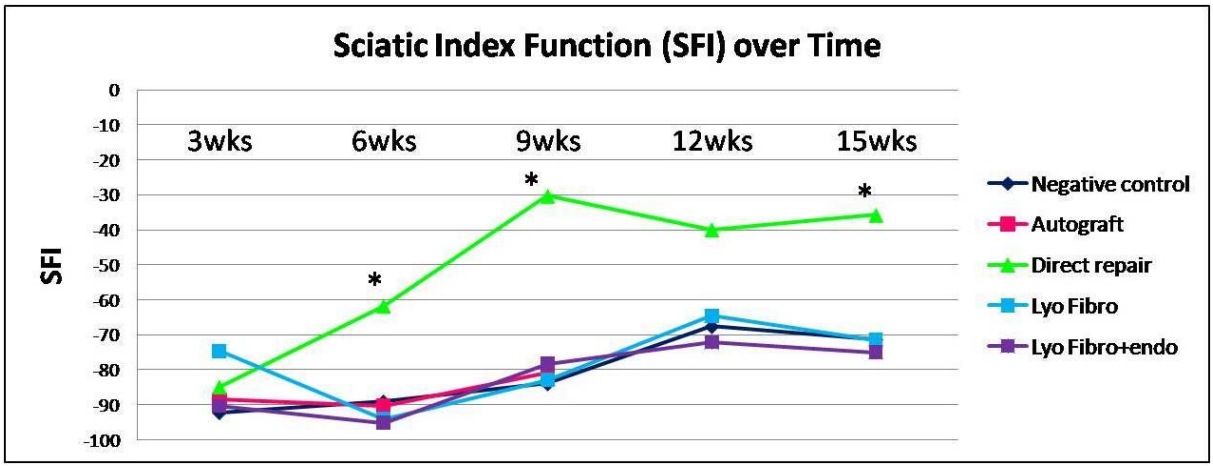

Figure 4.23 Sciatic Function Index over Time in the Different Experimental Groups (*=$p<0.005$)

The re-explored nerve-tubes were in continuity in all specimens and there was no evidence of inflammatory reaction at the site of nerve-tube reapproximation.

The total number of myelinated axons present at the site of the graft and distal to the graft was assessed (Figure 4.24). Because of restrictions in financial resources and the absence of significant results in the experimental groups, the number of myelinated axons was not assessed in the direct repair group, which was mainly used as a positive control for the functional motor assessment. The autograft group served in this experiment as a positive control. There were significantly more myelinated axons present within the historic autograft group (1912.83±302.96) than within the lyophilized filled nerve tube without a capillary-like network group (322.00±276.37, p=0.005) or the lyophilized filled tube with a capillary-like network group (641.63±276.37, p=0.0313). There was no statistically significant difference between the number of myelinated axons present distal to the graft (p=0.1196). However, some specimen of the experimental groups definitively showed signed of axonal regeneration (Figure 4.25).

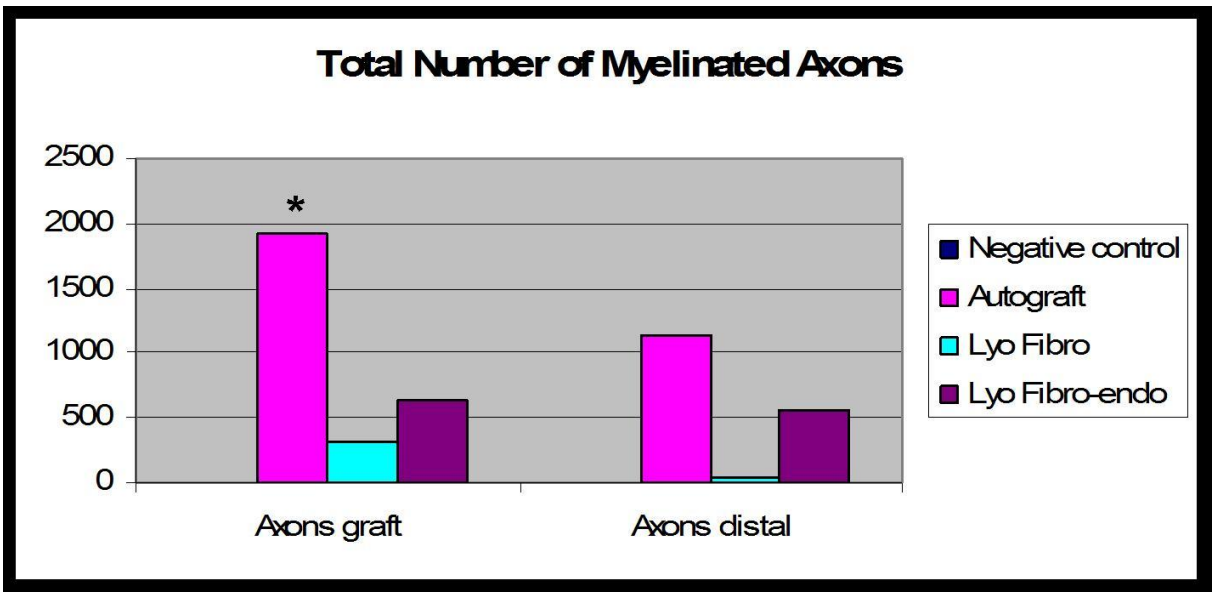

Figure 4.24 Number of Myelinated Axons within the Different Experimental Groups (*=p<0.05 with all other experimental groups).

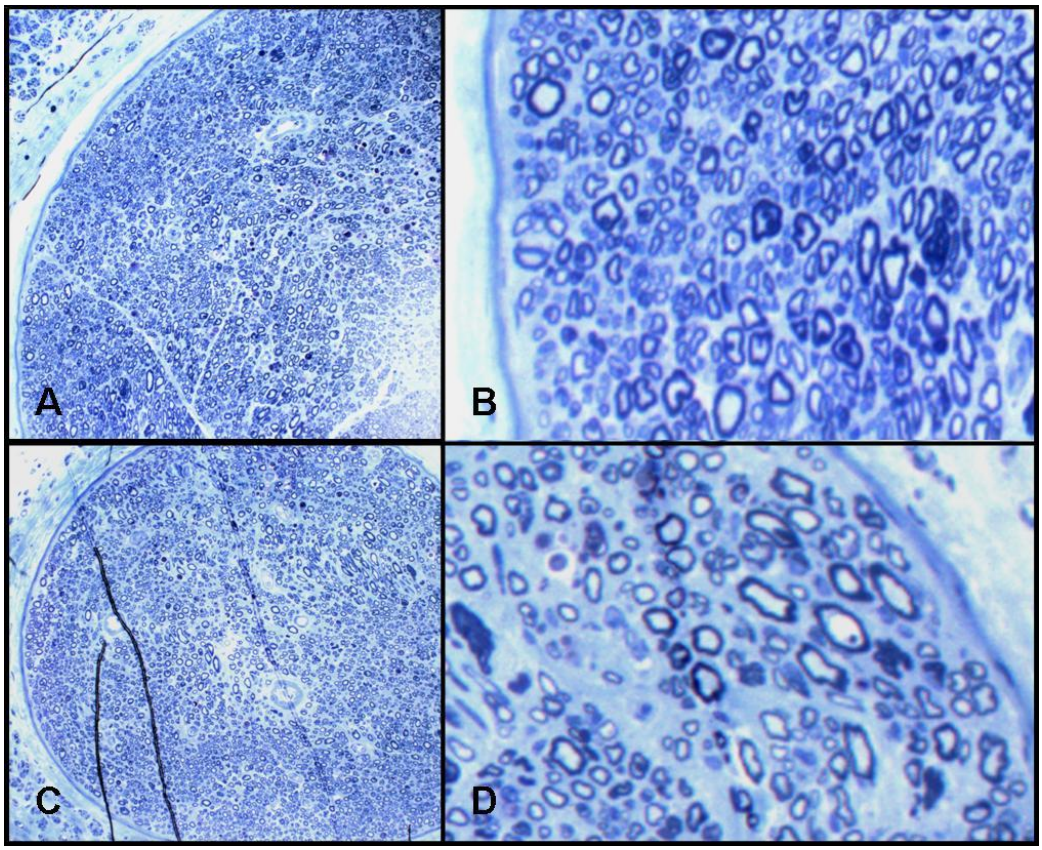

Figure 4.25 Axonal Regeneration within Filled Tissue-Engineered Nerve Tubes
Semi-thin sections (1um) stained with toluidine blue demonstrating regenerating axons within the graft segments (A, B) and the distal sciatic nerve (C, D).

The ratio of the experimental versus control gastrocnemius weight (Figure 4.26) was significantly different ($p<0.0001$) between the direct repair group (0.66 ± 0.02) and the sciatic nerve resection group (0.16 ± 0.02), the lyophilized filled nerve tube without a capillary-like network group (0.14 ± 0.01) as well as with the lyophilized filled nerve tube with a capillary-like network group (0.15 ± 0.01). Similar statistically significant ($p<0.0001$) differences between these later 3 groups were also present with the autograft group weight ratio (0.61 ± 0.02). There was no statistically significative differences between the filled tubes groups ($p=0.95$) or between the autograft and direct repair groups ($p=0.21$).

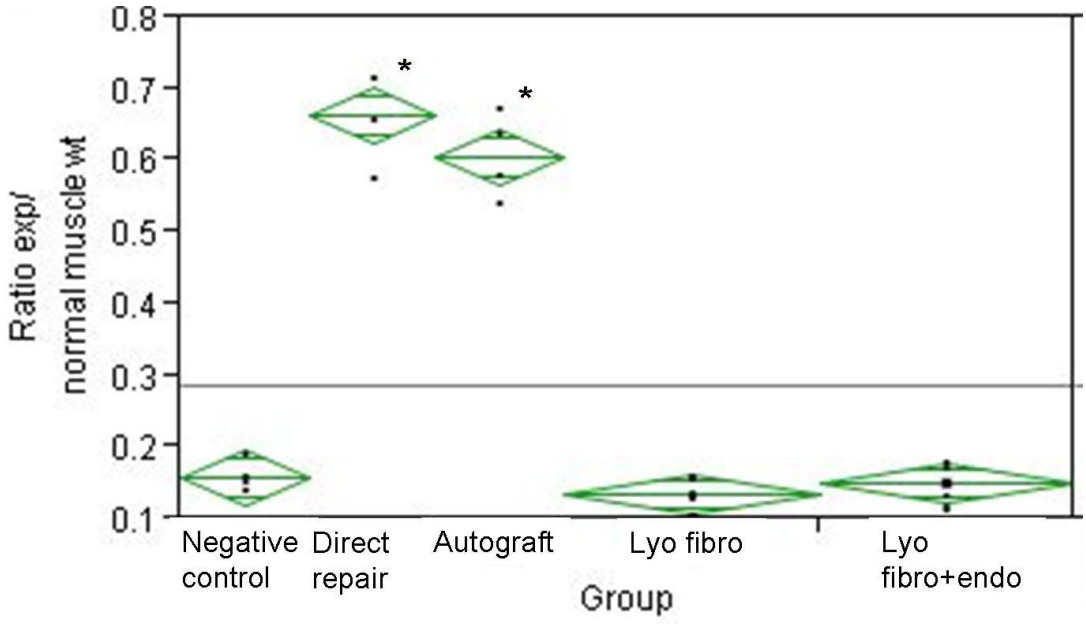

Figure 4.26 Relative Gastrocnemius Muscle Weight between the Experimental Groups (*=p<0.0001)

CHAPTER 5 - DISCUSSION

CHAPTER 5. DISCUSSION

The main aim of this thesis was to evaluate the potential to develop a tubular biomaterial supporting nerve regeneration in the context of peripheral nerve trauma, using tissue-engineering approaches. The goal was to initiate the design of reconstructed, completely biological, nerve guides from autologous or heterologous cells, with or without an internal capillary-like network. This approach for the development of a nerve guide is novel and, to our knowledge, has not been explored by other groups. Biological conduits have been studied, including arteries[61], veins[23,130,140], skeletal muscles[10,61], and epineural sheaths[67,117]. Attempts are made to modify them and improve their capacity to support nerve regeneration. For example, a combined conduit where a vein graft is filled with muscle has been proposed[19], studied, and applied clinically in a small series of patients[11]; an epineural conduit augmented with different supportive cell therapies is also being investigated[67,117]. However, the possible modifications of these biological structures are somewhat limited. Therefore, tissue-engineering methods are explored by multiple groups to create artificial nerve conduits that could support and ideally improve nerve regeneration. This approach allows manipulation of multiple factors, including the mechanical properties, the permeability, the internal architecture, and the supporting additional elements (cells or neurotrophic factors) of these guides. This project's approach combines the tissue-engineering techniques, which have the possibility to manipulate the components into producing a conduit with an appropriate design, with a completely biological approach, the exclusive use of human cultured cells.

Previously, the LOEX has designed human fibroblast-based nerve tubes using tissue-engineered approaches, but these tubes were hollow. However, there is more and more evidence that a simple tubular structure cannot support optimal nerve regeneration[12,33]. It is generally thought that bridging long peripheral nerve gaps requires nerve tubes with an internal architecture that promotes regeneration by supporting and directing axonal migration[12,66]. With this rationale, the hollow fibroblast tubes were filled with a chitosan-collagen gel to provide an internal framework for axonal regeneration. Chitosan, a polysaccharide obtained from N-deacetylation of chitin, is biodegradable and biocompatible[106]. Nerve guides produced with chitosan, functionalized with growth factors and laminin, enhanced functional and sensory

recovery when tested in vivo[102]. Chitosan conduits filled with collagen sponge supplemented with nerve growth factor have also been shown to be equivalent to autograft in vivo[45]. Similarly, collagen filaments have been found to guide axonal regeneration[95,144]. Moreover, collagen proteins share a triple helical structure due to a conserved primary sequence (i.e. a glycine residue in every third position of their polypeptide chain)[22]. This repeat structure, which characterizes all collagens, and their helical 3-dimensional structure generally display a weak antigenic activity[22]. For these reasons, the hollow tubes were filled with a chitosan-collagen gel to provide support for axonal regeneration.

The potential of these tissue-engineered nerve tubes (i.e. the hollow and tubes filled with a chitosan-collagen gel) to support nerve regeneration was tested using the rat sciatic nerve model. As described in the result section, both these tubes were unable to support axonal regeneration across a 15mm gap in the rat sciatic nerve model. This result can be explained first by the possible infection and/or host reaction to the tubes that was observed at the time of re-exploration at the end of the study period. The signs related to graft or nerve tube rejection are local and include a severe inflammatory response with a lymphocytic infiltrate, Schwann cell necrosis, and a disruption of the nerve tubes[94]. It is possible that an inflammatory process to the implanted nerve tubes prevented nerve regeneration. Another possible explanation for the negative results is the use of a 15mm nerve gap length. It is well known that in nerve gaps of more than 15mm in rats, the formation of the fibrin cable and of the Bands of Büngner is compromised and requires exogenous support[5,12,131]. The distal stump is not sufficient as a stimulus for nerve regeneration over this distance[78,79]. It is therefore expected that the hollow tissue-engineered nerve tube did not support nerve regeneration over a 15mm nerve gap. As for the tissue-engineered tubes filled with chitosan-collagen gel, axonal regeneration along the long nerve gap may have required the addition of nerve growth factors. Further experiments with tubes supplemented with nerve growth factors are being done (other LOEX investigator) to evaluate the ability of these tubes to sustain nerve regeneration. It is also possible that the tubes' 3-dimensional non-organized structure may not have been optimal to guide the regenerating axons. Growing axons are known to prefer two-dimensional structures to three-dimensional constructs[12,66]. The random orientation of the chitosan-collagen scaffold may not have guided the axons adequately. Many authors

have reported that nanofiber orientation influences cell growth[24,41,70,87,88] and that aligned nanofibers provide an effective contact guidance effect during neurite growth[22].

The chitosan-collagen gel is a natural polymer, but still carries a potential for inflammatory and/or immune or host reaction. Despite its relatively weak antigenic activity, anti-collagen antibodies can be produced by heterogenic collagen[34,116]. The risk, although very low, of viral or prion protein transmission via the bovine collagen is also theoretically present.

Therefore, attempts were made to see whether this chitosan-collagen gel could be replaced by an internal architecture made of human cells and of their extracellular matrix. Results indicate that tubes with a regular internal architecture can be formed from fibroblast sheets alone, i.e. the fibroblast cells and their extracellular matrix. A reliable and reproducible technique was developed as a part of this thesis. Rolling the fibroblast sheets along the reconstructed nerve guide axis without an inert tubular support creates tubes with an internal architecture. These tubes consist of 2-dimensional sheets longitudinally arranged in a 3-dimensional construct, the nerve tube. Theoretically, regenerating axons could migrate longitudinally along the 2-dimensional sheet structures. The concentric layers were sufficiently separated (20 to 40 µm) to allow the migration of unmyelinated (0.3 µm to 2.5 µm) and myelinated (1µm to 16 µm) nerve fibers[43]. When combined with the previously-designed hollow tube, the nerve guides were of sufficient stiffness to allow micro-suturing. The extracellular matrix composition of these tubes has all the ideal characteristics of a biomaterial: they are biocompatible, they contain structural and functional molecules, and they provide a supportive medium for blood vessels and for the diffusion of nutrients[7].

The tissue-engineered tubes could potentially be used as living constructs; the living cells could produce factors that would improve nerve regeneration. The study of this hypothesis would require testing living nerve tubes produced from rat fibroblast in the rat sciatic nerve model. With this rationale, an attempt was made to culture rat fibroblasts and produce rat fibroblast sheets that could be rolled into filled tubes with the technique developed in this thesis. With some modification of the culture parameters, rat fibroblasts producing sufficient extracellular matrix to allow manipulation was possible. The main factor influencing the formation of extracellular matrix from rat fibroblasts was

the length of time in culture; 70 days of culture were required for rat fibroblast. Other factors that were modified and may have influenced the extracellular matrix formation include culture medium supplementation with glutamine, cell passage, different initial seeding concentration, freshly extracted cells versus thawed cells, the area of origin of the skin fibroblasts, and the rat strain. However, the exact influence of each of these factors was not studied, as it was not the focus of this thesis. Despite the multiple trials, the sheets obtained were still much thinner and less resistant to manipulation than the ones obtained from human fibroblasts. To create rat filled tubes composed of fibroblasts, further modifications will be necessary. These modifications could either be in the production methods of the rat fibroblast sheets or in the techniques used for the formation of the filled tubes to accommodate for the fragility of the rat fibroblast sheets. One could consider adding insulin to the culture medium, as it was shown to strongly and significantly stimulate absolute collagen production in a dose-dependent manner[120,136]. Another option would be to assemble rat fibroblast sheets, similar to the techniques used to assemble sheets for production of tissue-engineered skin[75], prior to rolling them into filled or hollow nerve tubes.

On the other hand, the tissue-engineered nerve tubes could be lyophilized before use. Lyophilization is a process by which water is removed from the material by sublimation at low temperatures and low pressures[8]. It is commonly used to preserve biological graft tissues, such as bone[20,25,62], tendon[121,132] and commercially available biological materials[8]. Compared to the more commonly used thermal decellularization technique in which the structure of the extracellular matrix is typically damaged[113], lyophilization preserves the general physical structure of the material. Moreover, the bioactivity of the collagen matrix enriched by growth factors produced by fibroblasts and trapped by the glycoproteins within the extracellular matrix[13] is retained by lyophilization[53,57,73,93]. During scaffold degradation, these extracellular matrix growth factors such as vascular endothelial cell growth factor, basic fibroblast growth factor, and transforming growth factor beta will be released and exert their biologic effects[138,,52,54-56,89]. Lyophilization also has the advantage that non-autologous biologic materials may be used in humans without evidence of adverse immunologic outcomes[7]. For all these reasons, we elected to first study nerve regeneration within the newly-designed filled nerve guidance tubes that had been lyophilized.

The potential of the newly designed tissue-engineered nerve tubes to support nerve regeneration was tested in the rat sciatic nerve model. There were no statistically definitive positive results for axonal nerve regeneration within lyophilized filled tissue-engineered tubes with and without a capillary-like network. However, some evidence of nerve regeneration was present on histology, which was not reflected by the functional studies or the relative gastrocnemius muscle weight. It is possible that the tissue-engineered nerve tubes were too compact to allow nerve regeneration. The available space for the migration of Schwann cells and of regenerating axons may not have been sufficient. The technique used to produce these tubes will need to be optimized further to provide a better substrate to sustain nerve regeneration. It is possible that despite their 2-dimensional surface, the regenerating axons were not sufficiently directed. Small groove size (5μm) promotes significantly more parallel growth of neurites than larger grooves[143]. Since the tubes were lyophilized (decellularized), the fibroblasts that were used to produce the tubes cannot have further proliferated. However, if living constructs were implanted, this could be a legitimate concern.

It is also possible that the experimental model, i.e., the rat sciatic nerve model, and the assessment methods, i.e. the sciatic function index, Neurometer threshold, axon count, and relative gastrocnemius muscle weight, may not be optimal to test nerve regeneration. The positive control experimental groups (i.e., direct repair and autograft) showed data consistent with significant nerve regeneration only in the axon count and relative gastrocnemius muscle weight, but not consistently on the functional tests. Despite its disadvantages, the rat sciatic nerve model was chosen because it is widely used for the study of nerve regeneration in vivo[6]. The correlation between the three commonly utilized parameters (histomorphometry, electrophysiology, and walking track analysis) to evaluate nerve regeneration is only moderate[135]. In this thesis, only the direct repair experimental group showed a significative improvement in the sciatic function index, although both the direct repair and autograft experimental groups were associated with similar relative gastrocnemius muscle weight. The functional outcome measures of recovery (in this case the sciatic function index and the Neurometer threshold) ultimately represent the most important readouts, but their sensitivity would need to be optimized for future work. In this thesis, the functional analysis was in part limited by the formation of contractures. Physiotherapy, which was performed daily post-operatively, helps prevent contractures. However, part of the limitation of the functional analysis may have been

differential reinnervation of the tibial and peroneal portion of the sciatic nerve, creating a flexion contracture. It is possible that other nerve regeneration assessment methods, such as electrophysiology, video-assisted gait pattern analysis, more detailed histomorphometry, and/or retrograde labeling, may have helped in evaluating the nerve regeneration within our tissue-engineered nerve tubes. However, these techniques were not available within the laboratory at the time of this thesis.

One major hypothesis of this thesis was that a capillary-like network could be developed within the completely biological tissue-engineered tubes. Results indicate that endothelial cells can survive and form capillary-like network within the fibroblast sheets, and that these capillary-like networks persist as the sheets are rolled to form the tissue-engineered nerve tubes. The capillary-like tube formation is due, in part, to the secretion of vascular endothelial growth factor (VEGF) by fibroblasts [13]. The deposition of a rich extracellular matrix by living fibroblasts also appears necessary to promote capillary-like formation[13]. The conditions necessary for capillary-like tube formation are maintained in the rolled fibroblast sheets.

The extent and characteristics of this capillary-like network is variable depending of the methods utilized. The factors influencing the formation of this capillary-like network include the initial seeding concentration of the endothelial cells onto the fibroblast sheets, the assembly-technique of the fibroblast sheets prior to formation of the filled tubes, as well as the maturation time. It seems that long maturation times (30 days) after the formation of the tubes produced the capillary-like networks most similar to those found in nerves (Figure 4.17). These longitudinal capillary-like networks can be obtained with lower initial seeding concentration of endothelial cells if two fibroblast sheets are assembled before formation of the filled tubes, or with higher initial seeding concentration of endothelial cells if a single fibroblast sheet is used.

The ability to produce these capillary-like networks within the tissue-engineered nerve tubes is a major advantage for the design of large-diameter tubes. Passive diffusion is thought to be sufficient only for nerve grafts smaller than 2mm. If larger-diameter nerve guidance tubes are used, absence of vascularization creates a hypoxic environment that will impair axonal migration. The capillary-like structure produced in our tissue-engineered nerve tubes could connect rapidly to the host vasculature, reduce the hypoxic

time of the tube environment, and improve axonal migration. This process of vascularization by the process of inosculation was demonstrated in an endothelialized reconstructed skin implanted in the mouse[133]. Further study will be needed to determine if the capillary-like networks produced in the tissue-engineered nerve tubes would also connect with the host vasculature once implanted. Since the use of umbilical vein endothelial cell is not practical for clinical applications, it will also be necessary to evaluate if similar capillary-like network can be obtained from microvascular endothelial cells extracted from skin biopsy. Finally, it will be necessary to evaluate if indeed nerve regeneration is possible within large endothelialized nerve guide and if nerve regeneration is improved in smaller nerve guide.

Nerve guidance tubes with a capillary-like network could also be used once lyophilized, and this is why we evaluated their potential for nerve regeneration once lyophilized. In this situation, the capillary-like network would not be used to promote rapid vascularization. However, laminin, produced by endothelial cells in their basal membrane[58], is known to improve nerve regeneration[60,147]. Laminin is abundant component of the basement membrane during the development of the embryonic nervous system, but is also present in the mature nervous system, where it has an important function, that includes a role in guidance and adhesion[22]. The laminin, and other growth factors produced by endothelial cells and fibroblasts, enrich the collagen matrix and remain trapped by the glycoproteins within the extracellular matrix[13]. Including a capillary-like network within the tissue-engineered nerve guides could therefore promote nerve regeneration even if these tubes were used once lyophilized.

The time necessary to produce the filled guidance tubes with or without a capillary-like network, i.e., approximately 2 months, may be viewed as a problem. However, except for sharp nerve lacerations, most nerve injuries are observed to assess for possible spontaneous recovery. This period of observation could be used to produce guidance tubes in cases for which the use of these tubes is very likely. In closed traction injuries, the observation period is generally from 3 to 6 months, which would give the necessary time for the production of guidance tubes. In non-sharp lacerations (e.g. saw, propeller blade, etc.), surgeons generally wait 3 to 4 weeks in order to assess intraoperatively the extent of the injury at both nerve stumps. In these patients, it could be reasonable to prolong this observation period by a few weeks. In cases requiring

surgery that are evaluated late, the use of nerve guidance tubes with live cells would not be possible; the time necessary for their production would delay the surgical repair which would then be associated with decreased surgical results. Injuries involving sensory nerves are different; return of function is not limited in time and sensory nerves can be repaired even after prolonged periods. In these cases, the time required to produce the guidance tubes would not be a limitation. It is also possible that the guidance tubes be used, in part or totally, as a lyophilized construct; the tubes could therefore be heterologous and ready for utilization. Finally, the ultimate goal is to produce a guidance tube that would be superior to the autograft; if possible, the time required to construct these guidance tubes may be justified.

The main objectives of this thesis were met. A tissue-engineering approach to produce reconstructed completely biological nerve guides from living autologous or heterologous cells was developed. Even though these newly-designed tissue-engineered nerve grafts were not shown to support nerve regeneration within the rat sciatic nerve model, some evidence of axonal regeneration was present. Further modification in the architecture of these tubes will likely produce a completely biological nerve guide able to sustain nerve regeneration. Finally, an internal capillary-like network resembling a native neural vascular network was developed within these tissue-engineered nerve grafts.

CHAPTER 6 - CONCLUSION

CHAPTER 6. FINAL CONCLUSION AND SUMMARY

The main aim of this thesis, which was to initiate the design of reconstructed, completely biological, nerve guides from autologous or heterologous cells, with or without an internal capillary-like network, was fulfilled. This project's approach was novel; it combined the tissue-engineering techniques with a completely biological approach. A technique was designed to create completely biological nerve guides from living autologous or heterologous cells with an internal architecture. Moreover, an internal capillary-like network resembling a native neural vascular network could be developed within these tissue-engineered nerve grafts.

Even though further studies are needed to optimize the internal architecture, the technique, and to test the tissue-engineered tube in vivo, these guidance nerve tubes represent an innovative approach for the development of an alternative to autograft. They could be used as living or lyophilized nerve guides, formed from autologous cells or well-characterized heterologous cell, and with or without a capillary-like network.

This thesis demonstrates that nerve guidance tube can be created with this innovative approach. These tissue-engineered nerve tubes could be applicable in clinical practice. Even if from autologous cells, only a small skin biopsy would be necessary, and the delay for culture time should not exceed 2 months, which is acceptable in patients presenting early after a nerve injury, during the observation period to assess for spontaneous regeneration, with the repair still occurring prior to 6 months. Moreover, nerve guides of various dimensions (length and diameter) could be produced, especially with rapid vascularization of the implanted tube by inosculation of a pre-existing capillary-like network.

REFERENCES

1. Adams DN, Kao EY, Hypolite CL, Distefano MD, Hu WS, Letourneau PC: Growth cones turn and migrate up an immobilized gradient of the laminin IKVAV peptide. J Neurobiol 62: 134–47, 2005

2. Adams WE: Blood supply of nerves. J Anat 76: 323-341, 1942

3. Aebischer P, Guenard V, Brace S: Peripheral nerve regeneration through blind-ended semipermeable guidance channels: effect of the molecular weight cutoff. J Neurosci 9: 3590-3595, 1989

4. Aebischer P, Guenard V, Valentini RF: The morphology of regenerating peripheral nerves is modulated by the surface microgeometry of polymeric guidance channels. Brain Res 531: 211-218, 1990

5. Ahmed Z, Brown RA: Adhesion, alignment, and migration of cultured Schwann cells on ultrathin fibronectin fibres. Cell Motil Cytoskeleton 42: 331–343, 1999

6. Bain JR, Mackinnon SE, Hunter DA : Functional evaluation of complete sciatic, peroneal and posterior tibial nerve lesions in the rat. Plast Reconstr Surg 83: 129-136, 1989

7. Badylak SF: The extracellular matrix as a biological scaffold material. Biomaterials 28: 3587-3593, 2007

8. Badylak SF, Freytes DO, Gilbert TW: Extracellular matrix as a biological scaffold material: Structure and function. Acta Biomateriala 5: 1-13, 2009

9. Batiston B, Genuna S, Ferrero M, Tos P: Nerve repair by means of tubulization: literature review and personal clinical experience comparing biological and synthetic conduits for sensory nerve repair. Microsurgery 25: 258-267, 2005

10. Battiston B, Tos P, Conforti LG, Geuna S: Alternative techniques for peripheral nerve repair. Conduits and end-to-side neurorrhaphy. Acta Neurochir Suppl 100: 43-50, 2007

11. Battiston B, Tos P, Cushway TR, Geuna S: Nerve repair by means of vein filled with muscle grafts I. Clinical results. Microsurgery 20: 32-36, 2000

12. Bellamkonda RV: Peripheral nerve regeneration: An opinion on channels, scaffolds and anisotropy. Biomaterials 27: 3515-3518, 2006

13. Berthod F, Germain L, Tremblay N, Auger FA: Extracellular matrix deposition by fibroblasts is necessary to promote capillary-like tube formation in vitro. J Cell Physiol 207: 491–498, 2006

14. Bertleff MJ, Meek MF, Nicolai JP: A prospective clinical evaluation of biodegradable Neurolac nerve guides for sensory nerve repair in the hand. J Hand Surg Am 30: 513-518, 2005

15. Black A, Berthod F, L'Heureux N, Germain L, Auger FA: In vitro reconstruction of a human capillary-like network in a tissue-engineered skin equivalent. FASEB J 12: 1331–1340, 1998

16. Black MM, Lasek RJ: Slow components of axonal transport: two cytoskeletal networks. J Cell Biol 86: 616-623, 1980

17. Braga-Silva J: The use of silicone tubing in the late repair of the median and ulnar nerves in the forearm. J Hand Surg Br 24: 703-706, 1999

18. Brink HE, Bernstein J, Nicoll SB: Fetal dermal fibroblasts exhibit enhanced growth and collagen production in two- and three-dimensional culture in comparison to adult fibroblasts. J Tissue Eng Regen Med 3: 623-33, 2009

19. Brunelli GA, Battiston B, Vigasio A, Brunelli G, Marocolo D: Bridging nerve defects with combined skeletal muscle and vein conduits. Microsurgery 14: 247-251, 1993

20. Burchardt H, Jones H, Glowczewskie F, Rudner C, Enneking WF: Freeze-dried allogenic segmental cortical-bone grafts in dogs. J Bone Joint Surg Am 60: 1082–90, 1978

21. Carr MM, Best TJ, Mackinnon SE, Evans PJ: Strain differences in autotomy in rats undergoing sciatic nerve transection or repair. Ann Plast Surg 28: 538-544, 1992

22. Chiono V, Tonda-Turo C, Ciardelli G: Artificial scaffolds for peripheral nerve reconstruction. Int Rev Neurobiol 87: 173-198, 2009

23. Chiu DT, Janecka I, Krizek TJ, Wolff M, Lovelace RE: Autogenous vein graft as a conduit for nerve regeneration. Surgery 91: 226-233, 1982

24. Christopherson GT, Song H, Mao HQ: The influence of fiber diameter of electrospun substrates on neural stem cell differentiation and proliferation. Biomaterials 30: 556-564, 2009

25. Cornu O, Banse X, Docquier PL, Luyckx S, Delloye C: Effect of freeze–drying and gamma irradiation on the mechanical properties of human cancellous bone. J Orthop Res 18: 426–31, 2000

26. Dahlin LB, Anagnostaki L, Lundborg G: Tissue response to silicone tubes used to repair human median and ulnar nerves. Scand J Plast Reconstr Surg Hand Surg 35: 29-34, 2001

27. Dellon AL: Use of silicone tube for reconstruction of a nerve injury. J Hand Surg Br 19: 271-272, 1994

28. Den Dunnen WFA, Meek MF: Sensory nerve function and auto-mutilation after reconstruction of various gap lengths with nerve guides and autologous nerve grafts. Biomaterials 22: 1171-1176, 2001

29. Den Dunnen W, van der Lei B, Robinson PH, Holwerda A, Pennings AJ, Schakenraad JM: Biological performance of a degradable poly(lactic acid-ε-caprolactone) nerve guide: influence of tube dimensions. J biomed Mater Res A 29: 757-766, 1995

30. de Ruiter GC, Onyeneho IA, Liang ET, Moore MJ, Knoght AM, Malessy MJ, Spinner RJ, Lu L, Currier BL, Yaszemski MJ, Windebank AJ: Methods for in vitro characterizaton of multichannel nerve tubes. J Biomed Mater Res A 84: 643-651, 2008

31. de Ruiter GCW, Spinner RJ, Yaszemski MJ, Windebank AJ, Malessy MJA: Nerve tubes for peripheral nerve repair. Neurosurg Clin N Am 20: 91-105, 2009

32. Dodla MC, Bellamkonda RV: Anisotropic scaffolds falicitate enhanced neurite extension *in vitro*. J Biomed Mater Res A 78: 213-221, 2006

33. Dumont CE, Born W: Stimulation of neurite outgrowth in a human nerve scaffold designed for peripheral nerve reconstruction. J Appl Biomater 73B: 194-202, 2005

34. Ellingsworth LR, De Lustro F, Brennan JE, Sawamura S, McPHerson J: The human immune response to reconstituted bovine collagen. J Immunnol 136: 877-882, 1986

35. Engh CA, Schofield BH: A review of the central response to peripheral nerve injury and its significance in nerve regeneration. J Neurosurg 37: 198-203, 1972

36. Evans GR: Peripheral nerve injury: a review and approach to tissue engineered constructs. Anat Rec 263: 396–404, 2001

37. Evans PJ, Mackinnon SE, Best TJ, Wade JA, Awerbuck DC, Makino AP, Hunter DA, Midha R: Regeneration across preserved peripheral nerve grafts. Muscle Nerve 18: 1128-1138, 1995

38. Evans GbK, Widmer M, Gürlek A, et al: Tissue engineered conduits: the use of biodegradable poly-DL-lactic-co-glycolic acid (PLGA) scaffolds in peripheral nerve regeneration. Berlin (Germany): Springer, 1998

39. Fu SY, Gordon T: The cellular and molecular basis of peripheral nerve regeneration. Mol Neurobiol 14: 67–116, 1997

40. Gerhart D, Drewes L: Glucose transporters at the blood-nerve barrier are associated with perineurial cells and endoneurial microvessels. Brain Res 508: 46-50, 1990

41. Ghasemi-Mobarakeh L, Prabhakaran MP, Morshed M, Nasr-Esfahani MH, Ramakrishna S: Electrospun poly(ε-caprolactone)/gelatin nanofibrous scaffolds for nerve tissue engineering. Biomaterials 29: 4532-4539, 2008

42. Gomez TM, Letourneau PC: Filopodia initiate choices made by sensory neuron growth cones at laminin/fibronectin borders in vitro. J Neurosci 14: 5959–5972, 1994

43. Guena S, Raimondo S, Ronchi G, Di Scipio F, Tos P, Czaja K, Fornaro M: Histology of the peripheral nerve and changes occurring during nerve regeneration. Int Rev Neurobiol 87: 27-46, 2009

44. Guenard V, Valentini RF, Aebischer P: Influence of surface texture of polymeric sheets on peripheral nerve regeneration in a two-compartment guidance system. Biomaterials 12: 259-263, 1991

45. Guo BF, Dong MM: Application of neural stem cells in tissue-engineered artificial nerve. Otolaryngol Head Neck Surg 140: 159-164, 2009

46. Guttman E, Young JZ: Reinnervation of muscle after various periods of atrophy. J Anat 78: 15-43, 1944

47. Hadlock TA, Sundback CA, Hunter DA, Vacanti Jp, Cheney ML: A new artificial nerve graft containing rolled Schwann cell monolayers. Microsurgery 21: 96-101, 2001

48. Hall S: Nerve repair: A neurobiologist's view. J Hand Surg 26B: 129-136, 2001

49. Hall S: The response to injury in the peripheral nervous system. J Bone Joint Surg Br 87: 1309-1319, 2005

50. Hare GMT, Evans PJ, Mackinnon SE, Best TJ, Bain JR, Szalai JP, Hunter DA: Walking track analysis: A long-term assessment of peripheral nerve recovery. Plast Reconstr Surg 89: 251-158, 1992

51. Henry EW, Chiu TH, Nyilas E, Brushart TM, Dikkes P, Sidman RL: Nerve regeneration through biodegradable polyester tubes. Exp Neurol 90: 652-676, 1985

52. Hodde J, Hiles M: Bioactive FGF-2 in sterilized extracellular matrix. Wounds 13: 195-201, 2001

53. Hodde J, Janis A, Ernst D, Zopf D, Sherman D, Johnson C: Effects of sterilization on an extracellular matrix scaffold: Part I. Composition and matrix architecture. J Mater Sci: Mater Med 18: 537-543, 2007

54. Hodde JP, Record RD, Liang HA, Badylak SF: Vascular endothelial growth factor in porcine-derived extracellular matrix. Endothelium 8: 11–24, 2001

55. Hodde J, Record R, Tullius B, Badylak S: Fibronectin peptides mediate HMEC adhesion to porcine-derived extracellular matrix. Biomaterials 23: 1841–8, 2002

56. Hodde JP, Record RD, Tullius RS, Badylak SF: Retention of endothelial cell adherence to porcine-derived extracellular matrix after disinfection and sterilization. Tissue Eng 8: 225–34, 2002

57. Horowitz B, Lippin A, Chang MY, Shulman RW, Vandersande J, Stryker MH, Woods KR: Preparation of antihemophilic factor and fibronectin from human plasma cryoprecipitate. Transfusion 24: 357-362, 1984

58. Hudon V, Berthod F, Black AF, Damour O, Germain L, Auger FA: A tissue-engineered endothelialized dermis to study the modulation of angiogenic and angiostatic molecules on capillary-like tube formation in vitro. Br J Dermatol 148: 1094–1104, 2003

59. Hudson TW, Evans GR, Schmidt CE: Engineering strategies for peripheral nerve repair. Clin Plast Surg 26: 617–628, 1999

60. Ijkema-Paassen J, Jansen K, Gramsbergen A, Meek MF: Transection of peripheral nerves, bridging strategies and effect evaluation. Biomaterials 25: 1583-1592, 2004

61. Itoh S, Shinomiya K, Samejima H, Ohta T, Ishizuki M, Ichinose S: Experimental study on nerve regeneration through the basement membrane tubes of the nerve, muscle, and artery. Microsurgery 17: 525-534, 1996

62. Jackson DW, Grood ES, Wilcox P, Butler DL, Simon TM, Holden JP: The effects of processing techniques on the mechanical properties of bone–anterior cruciate ligament–bone allografts. An experimental study in goats. Am J Sports Med 16: 101–5, 1988

63. Jenq CB, Coggeshall RE: Nerve regeneration through holey silicone tubes. Brain Res 361: 233-241, 1985

64. Jenq CB, Coggeshall RE: Permeable tubes increase the length of the gap that regenerating axons can span. Brain Res 408: 239-242, 1987

65. Jenq CB, Jenq LL, Coggeshall RE: Nerve regeneration changes with filters of different pore size. Exp Neurol 97: 662-671, 1987

66. Kannan RY, Salacinski HJ, Butler PEM, Seifalian AM: Artificial nerve conduits in peripheral nerve repair. Biotechnol Appl Biochem 41: 193–200, 2005

67. Karacaoglu E, Yuksel F, Peker F, Guler MM: Nerve regeneration through an epineurial sheath: Its functional aspect compared with nerve and vein grafts. Microsurgery 21: 196-201, 2001

68. Kato A, Takahashi H, Takahashi Y, Matsushime H: Inactivation of the cyclin D-dependent kinase in the rat fibroblast cell line, 3Y1, induced by contact inhibition. J Biol Chem 272: 8065-8070, 1997

69. Kim DH, Connolly SE, Zhao S, Beuerman RW, Voorhies RM, Kline DG: Comparison of macropore, semipermeable, and nonpermeable collagen conduits in nerve repair. J Reconst Microsurg 9: 415-420, 1993

70. Kim TG, Lee DS, Park TG: Controlled protein release from electrospun biodegradable fiber mesh composed of poly(ε-caprolactone) and poly(ethylene oxide). Int J Pharm 338: 276-283, 2007

71. Kline DG, Selected basic considerations, In: Kline & Hudson's Nerve Injuries, Operative Results for Major Nerve Injuries, Entrapments, and Tumors, Second edition, Saunders, Elsevier, Philadelphia, 2008

72. Koga K, Furue H, Rahid MH, Takaki A, Katafuchi T, Yoshimura M: Selective activation of primary afferent fibers evaluated by sine-wave electrical stimulation. Molecular Pain 1: 13, 2005

73. Kolenik SA, McGovern TW, Leffell DJ: Use of a lyophilized bovine collagen matrix in postoperative wound healing. Dermatol Surg 25: 303-307, 1999

74. Koshimune M, Takamatsu K, Nakatsuka H, Inui K, Yamano Y, Ikada Y: Creating bioabsorbable Schwann cell coated conduits through tissue engineering. Biomed Mater Eng 13: 223–229, 2003

75. L'Heureux N, Pâquet S, Labbé R, Germain L, Auger FA: A completely biological tissue-engineered human blood vessel. FASEB J 12: 47-56, 1998

76. Lundborg G: Intraneural microcirculation. Othop Clin North Am 19: 1-12, 1975

77. Lundborg, G: Structure and function of the intraneural microvessels as related to trauma, edema formation, and nerve function. J Bone Joint Surg 57: 938–948, 1975

78. Lundborg G, Dahlin LB, Danielsen N, Gelberman RH, Longo FM, Powell HC, Varon S: Nerve regeneration in silicone chambers: influence of gap length and of distal stump components. Exp Neurol 76: 361-375, 1982

79. Lundborg G, Dahlin LB, Danielson N, Hansson HA, Johannesson A, Longo FM, VAron S: Nerve regeneration across an extended gap: a neurobiological view of nerve repair and possible neuronotrophic factors. J Hand Surg Am 7: 580-587, 1982

80. Lundborg G, Rosen B, Dahlin L, Danielsen N, Holmberg J: Tubular versus conventional repair of median nerve and ulnar nerves in the human forearm: early results from a prospective, randomized, clinical study. J Hand Surg Am 22: 99-106, 1997

81. Lundborg G, Rosen B, Dahlin L, Holmberg J, Rosen I: Tubular repair of the median or ulnar nerve in the human forearm: a 5-year follow-up. J Hand Surg Br 29: 100-107, 2004

82. Lundborg G, Rydevik B: Effects of stretching the tibial nerve of the rabbit. A preliminary study of the intraneural circulation and the barrier function of the perineurium. J Bone Joint Surg 55B:390-401, 1973

83. MackinnonSE, Dellon AL: Clinical nerve reconstruction with a bioabsorbable polyglycolic acid tube. Plast Reconstr Surg 85: 419-424, 1990

84. Mackinnon SE, Dellon AL, Hudson AR, Hunter DA: Alteration of neuroma formation by manipulation of its microenvironment. Plast Reconstr Surg 76: 345-353, 1985

85. Mackinnon SE, Doolabh VB, Novak CB, Trulock EP: Clinical outcome following nerve allograft transplantation. Plast Reconstr Surg 107: 1419-1429, 2001

86. Madisson RD, da Silva C, Dikkes P, Sidman RL, Chiu TH: Peripheral nerve regeneration with entubulation repair: comparison of biodegradable nerve guides versus polyethylene tubes and the effects of a laminin-containing gel. Exp Neurol 95: 378-90, 1987

87. Mahoney MJ, Chen RR, Tan J, Saltzman WM: The influence of microchannels on neurite growth and architecture. Biomaterials 26:771-778, 2005

88. Matsumoto K, Ohnishi K, Sekine T, Udea H, Yamamoto Y, Kiyotani T, Nakamura T, Endo K, Shimizu Y: Use of a newly developed artificial nerve conduit to assist peripheral nerve regeneration across a long gap in dogs. ASAIO J 46:415-420, 2000

89. McDevitt CA, Wildey GM, Cutrone RM: Transforming growth factor-beta1 in a sterilized tissue derived from the pig small intestine submucosa. J Biomed Mater Res 67A: 637–40, 2003

90. Medinaceli L, Freed WJ, Wyatt RJ: An index of the functional condition of rat sciatic nerve based on measurements made from walking tracks. Exp Neurol 77: 634-643, 1982

91. Meek MF, Coert JH, Hermens RA, Nicolai JP: Letter to the editor: The use of silicone tubing in the late repair of the median and ulnar nerves in the forearm. J Hand Surg Br 25: 408-409, 2000

92. Merle M, Dellon AL, Campbell JN, Chang PS: Complications from silicon-polymer intubulation of nerves. Microsurgery 10: 130-133, 1989

93. Mian E, Mian M, Beghe F: Lyophilized type-I collagen and chronic leg ulcers. Int J Tissue React 13: 257-269, 1991

94. Midha R, Mackinnon SE, Evans PJ, Best TJ, Hare GM, Hunter DA, Falk-Wade JA: Comparison of regeneration across nerve allografts with temporary or continuous cyclosporin A immunosuppression. J Neurosurg 78: 90-100, 1993

95. Millesi H: Techniques for nerve grafting. Hand Clin 16: 73-91, 2000

96. Mohanna PN, Terenghi G, Wiberg M: Composite PHB-GGF conduit for long nerve gap repair: A long-term evaluation. Scand J Plast Reconstr Surg Hand Surg 39: 129-137, 2005

97. Mosahebi A, Wiberg M, Terenghi G: Addition of fibronectin to alginate matrix improves peripheral nerve regeneration in tissue-engineered conduits. Tissue Eng 9: 209-217, 2003

98. Navarro X, Butí M, Verdú E: Autotomy prevention by amitriptyline after peripheral nerve section in different strains of mice. Rest Neurol Neurosci 6: 151-157, 1994

99. Nishimura A, Ogura T, Hase H, Makinodan A, Hojo T, Katsumi Y, Yagi K, Mikami Y, Kubo T: A correlative electrophysiologic study of nerve fiber involvement in carpal tunnel syndrome using current perception thresholds. Clin Neurophysiol 115: 1921–1924, 2004

100. Noble J, Munro CA, Prasad VS, Midha R: Analysis of upper and lower extremity peripheral nerve injuries in a population of patients with multiple injuries. J Trauma 45: 116-122, 1998

101. Ochoa J: Microscopic anatomy of unmyelinated nerve fibers. In: Dyck, PJ, Thomas PK, Lambert EH Eds: Peripheral neuropathy. Philadelphia, WB Saunders 113-150, 1975

102. Patel M, Mao L, Wu B, Vandevord PJ: GDNF-chitosan blended nerve guides: a functional study. J Tissue Eng Regen Med 1: 360-367, 2007

103. Pitta MC, Wolford LM, Mehra P, Hopkin J: Use of Gore-Tex tubing as a conduit for inferior alveolar and lingual nerve repair: experience with 6 cases. J Oral Maxillofac Surg 59: 493-396, 2001

104. Phillips JB, Bunting SC, Hall SM, Brown RA: Neural tissue engineering: a self-organizing collagen guidance conduit. Tissue Eng 11: 1611-1617, 2005

105. Pogrel MA, McDonald AR, Kaban LB: Gore-Tex tubing as a conduit for repair of lingual and inferior alveolar nerve continuity defects: a preliminary report. J Oral Maxillofac Surg 56: 319-321, 1998

106. Rinaudo M: Chitin and chitosan: Properties and applications. Prog Polym Sci 31: 603-632, 2006

107. Rodriguez FJ, Gomez N, Perego G, Navarro X: Highly permeable polylactide-caprolactone nerve guides enhance peripheral nerve regeneration through long gaps. Biomaterials 20: 1489-1500, 1999

108. Rosner BI, Hang T, Tranquillo RT: Scwhann cell behavior in three-dimensional collagen gels: evidence for differential mechano-transduction and the influence of TGF-beta 1 in morphological polarization and differentiation. Exp Neurol 195: 81-91, 2005

109. Sanders FK, Young JZ: The influence of peripheral nerve connections on the diameter of regenerating nerve fibers. J Exp Biol 22: 203-212, 1946

110. Sato T, Neschadim A, Konrad M, Fowler DH, Lavie A, Medin JA: Engineered human tmpk/AZT as a novel enzyme/prodrug axis for suicide gene therapy. Mol Ther 15: 962-970, 2007

111. Scaravilli F: Regeneration of the perineurium across a surgically induced gap in a nerve encased in a plastic tube. J Anat 139: 411-424, 1984

112. Schlosshauer B, Dreesmann L, Schaller HE, Sinis N: Synthetic nerve guide implants in humans: a comprehensive survey. Neurosurgery 59: 740-747, 2006

113. Schmidt CE, Leach JB: Neural tissue engineering: Strategies for repair and regeneration. Annu Rev Biomed Eng 5: 293-347, 2003

114. Seckel BR, Chiu TH, Nyilas E, Sidman RL: Nerve regeneration through synthetic biodegradable nerve guides: regulation by the target organ. Plast Reconstr Surg 74: 173-181, 1984

115. Seddon H: Three types of nerve injury. Brain 66: 237-288, 1943

116. Siegle RJ, McCoy IP, Schade W, Swansan NA: Intradermal implantation of bovine collagen. Humoral responses associated with clinical reactions. Arch Dermatol 120: 183-187, 1984

117. Siemionow M, Brzezicki G: Current techniques and concepts in peripheral nerve repair. Int Rev Neurobiol 87: 141-172, 2009

118. Siemionow M, Sommez E: Nerve allograft transplantation: a review. J Reconstr Microsurg 23(8): 511-520, 2007

119. Shen ZL, Lassner F, Becker M, Walter GF, Bader A, Berger A: Viability of cultured nerve grafts: an assessment of proliferation of Schwann cells and fibroblasts. Microsurgery 19: 356-363, 1999

120. Spanheimer RG: Inhibition of collagen production by diabetic rat serum: response to insulin and insulin-like growth factor-I added in vitro. Endocrinology 129: 3018–3026, 1991

121. Smith CW, Young IS, Kearney JN: Mechanical properties of tendons: changes with sterilization and preservation. J Biomech Eng 118: 56–61, 1996

122. Snow DM, Brown EM, Letourneau PC: Growth cone behavior in the presence of soluble chondroitin sulfate proteoglycan (CSPG), compared to behavior on CSPG bound to laminin or fibronectin. Int J Dev Neurosci 14: 331–49, 1996

123. Sporel-Özakat RE, Edwards PM, Hepgul KT, Savas A, Gispen WH: A simple method for reducing autotomy in rats after peripheral nerve lesions. J Neurosci Methods 36: 263-265, 1991

124. Stanec S, Stanec Z: Reconstruction of upper-extremity peripheral-nerve injuries with ePTFE conduits. J Reconstr Microsurg 14: 227-232, 1998

125. Sunderland S: A classification of peripheral nerve injuries producing loss of function. Brain 74: 491-516, 1951

126. Sunderland S. Nerve grafting. Edinburgh, London, New York: Churchill, Livingstone; 1978

127. Sunderland S, Bradley K: The cross-sectional area of peripheral nerve trunks devoted to nerve fibers. Brain 72: 428-439, 1949

128. Sunderland S, Ray LJ: Denervation changes in muscle. J Neurol Neurosurg Psychiatry 13: 159-177, 1950

129. Suzuki M, Itoh S, Yamaguchi I, Takakuda K, Kobayashi H, Shinomiya K, Tanaka J: Tendon chitosan tubes covalently coupled with synthesized laminin peptides facilitate nerve regeneration in vivo. J Neurosci Res 72: 646-659, 2003

130. Tang J, Wang XM, Hu J, Luo E, Qi MC: Autogenous standard versus inside-out vein graft to repair facial nerve in rabbits. Chin J Traumatol 11:104-109, 2008

131. Tong XJ, Hirai K, Shimada H, Mizutani Y, Izumi T, Toda N, Yu P: Sciatic nerve regeneration navigated by laminin-fibronectin double coated biodegradable collagen grafts in rats. Brain Res 663: 155–162, 1994

132. Toritsuka Y, Shino K, Horibe S, Nakamura N, Matsumoto N, Ochi T: Effect of freeze-drying or gamma-irradiation on remodeling of tendon allograft in a rat model. J Orthop Res 15: 294–300, 1997

133. Tremblay PL, Hudon V, Berthod F, Germain L, Auger FA: Inosculation of tissue-engineered capillaries with the host's vasculature in a reconstructed skin transplanted on mice. Am J Transplant 5: 1002–1010, 2005

134. Uzman BG, Villegas GM: Mouse sciatic nerve regeneration through semipermeable tubes: a quantitative model. J Neurosci Res 9: 325-338, 1983

135. Varejão ASP, Meek MF, Ferreira AJA, Patricio JAB, Cabrita AMS: Functional evaluation of peripheral nerve regeneration in the rat: walking track analysis. J Neurosci Methods 108: 1-9, 2001

136. Verhofstad MHJ, Bisseling TM, Haans EMH, Hendriks T: Collagen synthesis in rat skin and ileum fibroblasts is affected differently by diabetes-related factors. Int J Exp Path 79: 321-328, 1998

137. Vleggeert-Lankamp CL, de Ruiter GC, Wolfs JF, Pego AP, van den Berg RJ, Feirabend HK, Malessy MJ, Lakke EA: Pores in synthetic nerve conduits are beneficial to regeneration. J Biomed Mater Res A 80: 965-982, 2007

138. Voytik-Harbin SL, Brightman AO, Kraine MR, Waisner B, Badylak SF: Identification of extractable growth factors from small intestinal submucosa. J Cell Biochem 67: 478–91, 1997

139. Wall PD, Scadding JW, Tomkiewicz MM: The production and prevention of experimental anesthesia dolorosa. Pain 6: 175-182, 1979

140. Walton RL, Brown RE, Matory WE, Borah GL, Dolph JL: Autogenous vein graft repair of digital nerve defects in the finger: a retrospective clinical study. Plast Reconstr Surg 84: 944-949, 1989

141. Weber RA, Breidenbach WC, Brown RE, Jabaley ME, Mass DP: A randomized prospective study of polyglycolic acid conduits for digital nerve reconstruction in humans. Plast Reconstr Surg 106: 1036-1045, 2000

142. Weiss P: The technology of nerve regeneration: a review. Sutureless tubulation and related methods for nerve repair. J Neurosurg 1: 400-450, 1944

143. Yao I, Wang S, Cui W, Sherlock R, O'Connell C, Damodaran G, Gorman A, Windebank A, Pandit A: Effect of functionalized micropatterned PLGA on guided neurite growth. Acta Biomater 5: 580-588, 2009

144. Yoshii S, Oka M, Shima M, Taniguchi A, Akagi M: 30mm regeneration of rat sciatic nerve along collagen filaments. Brain Res 949: 202-208, 2002

145. Yoshimitsu M, Sato T, Tao K, Walia JS, Rasaiah VI, Sleep GT, Murray GJ, Poeppl AG, Underwood J, West L, Brady RO, Medin JA: Bioluminescent imaging of a marking transgene and correction of Fabry mice by neonatal injection of recombinant lentiviral vectors. Proc Natl Acad Sci USA 101, 16909-16914, 2004

146. Young BL, Begovac P, Stuart DG, Gloslow GE Jr: An effective sleeving technique in nerve repair. J Neurosci Methods 10: 51-58, 1984

147. Yu TT, Shoichet MS: Guided cell adhesion and outgrowth in peptide-modified channels for neural tissue engineering. Biomaterials 26: 1507-1514, 2005

148. Yu X, Bellamkonda RV: Tissue-engineered scaffolds are effective alternatives to autografts for bridging peripheral nerve gaps. Tissue Eng 9: 421-430, 2003

149. Zellem RT, Miller DW, Kenning JA, Hoening EM, Buchheit WA: Experimental peripheral nerve repair: Environmental control directed at the cellular level. Microsurgery 10: 290-301, 1989