

**EFFECTS OF EARLY AND DELAYED LASER
APPLICATION ON NERVE REGENERATION**

by

Tüba Akgül

B.S., Biology, Marmara University, 2008

Submitted to the Institute of Biomedical Engineering
in partial fulfillment of the requirements
for the degree of
Master of Science
in
Biomedical Science

Boğaziçi University

2012

**EFFECTS OF EARLY AND DELAYED LASER
APPLICATION ON NERVE REGENERATION**

APPROVED BY:

Assoc. Prof. Dr. Halil Özcan Gülçür
(Thesis Advisor)

Assoc. Prof. Dr. Murat Gülsoy
(Thesis Co-advisor)

Prof. Dr. Albert Güveniş

Asst. Prof. Dr. Bora Garipcan

Prof. Dr. Kemal Nuri Özerkan

DATE OF APPROVAL: 18 June 2012

ACKNOWLEDGMENTS

I would like to thank my supervisor Assoc. Prof. Dr. Halil Özcan Gülçür for his help and encouragement. Also, I would like to thank Assoc. Prof. Dr. Murat Gülsoy for his motivation and his comments during the time of this study.

I would like to thank Prof. Dr. Albert Güveniş, Prof. Dr. Kemal Nuri Özerkan and Asst. Prof. Dr. Bora Garipcan for being a member of my thesis committee and their valuable suggestions.

I am grateful to Assoc. Prof. Dr. Eyüp Kuşak for the dead-weight machine and his technical support. I must add Vet. Arzu Temizyürek for her invaluable contributions and help that made experiments possible.

I would like to express special thanks to my family for their support and patience during my education life. I also want to thank my friends, Betül Polat, Ayşegül Şen, Pınar Özel, İsmail Devecioğlu and Çağlar Gök for their assistance and encouragement during this work.

ABSTRACT

EFFECTS OF EARLY AND DELAYED LASER APPLICATION ON NERVE REGENERATION

Studies to understand the operating mechanism of the nervous system and to find new treatments for its diseases have been growing at an increased pace.

To accelerate the rate of regeneration, the laser is used immediately after surgery and the protocols in literature are generally adapted to this method. After crushing or transection of nerve, the mononuclear cells invade the injured segment and remove injured nerve structure. This degenerative event takes place at the first week after injury. These two critical points gave rise to the question of this study; are there any differences between early and delayed application? In this regard, three experimental groups underwent surgery (sciatic nerve was damaged by crushing for 10 minutes by applying a force of 50 N using dead-weight machine) and two of which were administered 14-days of low dose laser irradiation. 650 nm, 25 mW laser irradiation in continuous mode was applied to the early group immediately after injury whereas the therapy started one week after in the delayed group. The healing process of the damaged sciatic nerve has been shown to be accelerated in the laser therapy groups by means of functional, electrophysiological and histological examinations. It was observed that the sciatic functional index (SFI) value of the laser groups approximately reached to the normal whereas the SFI of the control group did not in day 21. However, this finding was not significantly important ($p > 0.05$). In addition, it was observed that the latency of the Compound Action Potential (CAP) decreased significantly ($p < 0.05$) in the delayed group. Moreover, histological examinations showed that the number of mononuclear cells was lower ($p < 0.05$) in the laser groups.

Although further tests are needed to be more conclusive, these results indicate that the laser therapy accelerates the rate of recovery. Both laser groups had positive results. However, the delayed group showed better recovery. This result may be due to the degeneration during the first-week.

Keywords: nerve regeneration, laser, sciatic functional index

ÖZET

ERKEN VE GEÇ LASER UYGULAMANIN SİNİR YENİLENMESİNE ETKİSİ

Sinir sistemi çalışma mekanizmasını anlama ve sinir sistemi hastalıklarına yönelik tedavi yöntemleri üzerine çalışmalar büyük hız kazanmıştır.

Sinir yenilenmesini hızlandırmak için kullanılan laser, zedelenmeden hemen sonra uygulanmaktadır ve literatürdeki protokollerin genel olarak bu yöntemi kullanmaktadır. Sinir kopması veya zedelenmesinden sonra, mononükleer hücreler zedelenmiş bölgeye akın ederek bu bölgeyi ortadan kaldırır. Bu süreç hasardan sonraki ilk bir hafta içerisinde gerçekleşir. Bu iki önemli bilgi şu sorunun doğmasına neden olmuştur; erken ve geç laser uygulamaları arasında herhangi bir fark var mı? Bu bağlamda, üç deneysel grupta hasar meydana getirilmiş (siyatik sinir 50 N uygulayan bir alet yardımıyla 10 dakika ezilmiştir) ve iki gruba 14 gün boyunca düşük doz laser uygulanmıştır. 650 nm 25 mW'lık devamlı kipteki laser erken grubuna hasardan hemen sonra, geç grubuna ise hasardan 1 hafta sonra uygulanmıştır. Fonksiyonel, elektrofizyolojik ve histolojik gözlemler yardımıyla laser uygulanan gruplarda zedelenen sinirin iyileşme sürecinin hızlandığı gösterilmiştir. 21.günde, laser grubunun SFI değerleri normale yaklaşırken, kontrol grubu yaklaşmadığı gözlemlenmiştir. Ancak bu bulgu istatistiksel olarak anlamlı değildi ($p>0.05$). Ayrıca, geç grubun Bileşik aksiyon potansiyel ait latans değerinin önemli ölçüde azaldığı gözlemlendi ($p<0.05$). Son olarak kullanılan histolojik gözlemler ise laser grubunda mononükleer hücre sayısının daha az olduğunu göstermiştir ($p<0.05$).

Her ne kadar kesin bir sonuca varmak için başka testlere gerek duyulsa da, elde edilen sonuçlar laserin iyileşme sürecini hızlandırdığını göstermektedir. İki laser grubunda da pozitif sonuçlar elde edilmiştir. Ancak, geç grup daha hızlı iyileşme göstermiştir. Bu sonuç ilk haftanın dejeneratif sürece ait olmasından kaynaklanabilir.

Anahtar Sözcükler: sinir yenilenmesi, laser, siyatik fonksiyonel indeks

TABLE OF CONTENTS

ACKNOWLEDGMENTS	iii
ABSTRACT	iv
ÖZET	v
LIST OF FIGURES	viii
LIST OF TABLES	x
LIST OF SYMBOLS	xi
LIST OF ABBREVIATIONS	xii
1. INTRODUCTION	1
1.1 Motivation	1
1.2 Objective	2
1.3 Outline	3
2. BACKGROUND	4
2.1 Structure of Peripheral Nerve	5
2.1.1 Endoneurium	5
2.1.2 Perineurium	6
2.1.3 Epineurium	6
2.2 Nerve Types and Structure	7
2.2.1 Myelinated Nerve Fiber	7
2.2.2 Unmyelinated Nerve Fiber	7
2.2.3 Action Potential	8
2.2.4 Axon Ultrastructure	9
2.3 Degeneration in PNS	10
2.4 Regeneration in The PNS	12
2.5 Laser in Repairing Injured Nerves	16
3. MATERIAL AND METHODS	17
3.1 Surgical Procedure	17
3.2 Functional Test: Sciatic Functional Index (SFI)	20
3.3 Electrophysiology	21
3.4 Histology	22

3.5	Statistical Analysis	24
4.	RESULTS	25
4.1	Functional Test: Sciatic Functional Index (SFI)	25
4.2	Electrophysiology	26
4.3	Histology	27
5.	DISCUSSION	30
5.1	The Experimental Set Up	30
5.2	The Effect of Laser on Inflammation	33
5.3	The Effect of Laser on Functional Recovery	33
5.4	The Effect of Laser on CAP Response	34
5.5	Possible Mechanism of Laser Effects	34
5.6	Recommendations for Future Work	35
	REFERENCES	37

LIST OF FIGURES

Figure 2.1	<p>Illustration of intact peripheral nerve structure of Wistar rat. a) The cross section of sciatic nerve; a: epineurium, b: perineurium, c: fascicle, d: tissue structure. 10X magnification, HE stain b) The longitudinal section of sciatic nerve. 40X magnification, HE stain.</p>	5
Figure 2.2	The Action Potential (AP)	8
Figure 2.3	The speed of nerve fibers	9
Figure 2.4	<p>Schematic of the main events of degeneration and regeneration after peripheral nerve injury. (A) Normal nerve fiber, maintaining synaptic contact with target cells. (B) Transection of the fiber results in distal fragmentation of axon and myelin sheaths. Macrophages and Schwann cells phagocytose degraded materials. Chromatolysis at the neuron soma and dendritic arbor retraction occur. (C) Fine sprouts emerge from the proximal axonal end, and elongate in association with the proliferated Schwann cells in the distal segment, that line up in bands of Büngner. (D) Axonal reconnection with target cells and maturation of the nerve fiber. The regenerated axon remains of smaller caliber and with shorter internodes than normal. The neuron returns to a normal transmitting phenotype. Target cells may suffer atrophy and phenotypic changes during denervation.</p>	15
Figure 3.1	The surgical procedure	18
Figure 3.2	The measurement of laser power with a detector	20
Figure 3.3	<p>(a) Walking Track. (b) EPL, ETS and EIT stand for the experimental paw lengths, the experimental toe spread (distance between the 1st and 5th toe) and the experimental intermediary toe spread (distance between the 2nd and 4th toe), respectively. NPL, NTS and NIT stand for the normal paw length, normal toe spread and normal intermediary toe spread, respectively.</p>	21

- Figure 4.1 Mean and standard deviation values of the SFI obtained from three groups at pre-surgery, the 7th day, the 14th day and the 21th day. 26
- Figure 4.2 Contralateral sciatic nerves are submitted to Normal groups. a) Mean latency of compound action potential. All groups are significantly different than Normal group ($p < 0.05$). There is also a significant difference between Delayed and Control groups ($p < 0.05$). b) Mean velocity of compound action potential. No significant difference between groups except that velocity of normal group is significantly different than other groups ($p < 0.05$). 27
- Figure 4.3 The number of mononuclear cells at the end of the study. All animals underwent crush lesion expect of Normal. No intervention in Control. Delayed and Early groups are submitted to laser phototherapy 7 days after surgery and immediately after surgery, respectively. The mean number of cells counted in images $10^4 \mu m^2$ captured at 400X. After 21 days, the number of mononuclear cells was significantly lower in both Delayed and Early groups than Control ($p < 0.05$, ANOVA and post hoc Tukey test). $p < 0.05$, in related to Normal group. $p < 0.05$, in related to Control group. 28
- Figure 4.4 a) Intact nerve b) Sciatic nerve crush lesion without any treatment. An increase in the number of mononuclear cells (arrows), myelin degeneration (*) c) Sciatic nerve of delayed group d) Sciatic nerve of early group. 29

LIST OF TABLES

Table 2.1	The speed of myelinated and unmyelinated axons	8
Table 2.2	Peripheral nerve injury classification done by Sunderland	11
Table 3.1	Groups of animals used in present investigation	19
Table 3.2	a) The steps of dehydration procedure	
	b) Summary of H&E staining steps	23
Table 4.1	Values obtained by Sciatic Functional Index mean \pm SD	25
Table 4.2	Velocity and Latency of Compound Action Potential	26

LIST OF SYMBOLS

α	Level of significance
p	Probability value
o	Degrees

LIST OF ABBREVIATIONS

AP	Action Potential
BDNF	Brain-derived neurotrophic factor
BNB	Blood Nerve Barrier
CAP	Compound Action Potential
CNS	Central Nervous System
CV	Conduction Velocity
DWM	Death-Weight Machine
g	gram
GAP-43	Growth-associated protein-43
HE	Hematoxylin-Eosin
He-Ne	Helium Neon
ITS	Intermediate total spread
J	Joule
Laser	Light amplification by stimulated emission of radiation
min	minute
mm	millimetre
MPa	Mega Pascal
N	Newton
NGF	Nerve growth
nm	nanometre
PL	Print Length
PNS	Peripheral Nervous System
Sc	Schwann cell
sec	second
SFI	Sciatic Functional Test
TS	Total Spread

1. INTRODUCTION

1.1 Motivation

Understanding the basic mechanisms of neural systems and finding cures for their disorders have become central issues in numerous researches. Obtaining fast and appropriate nerve regeneration after peripheral nerve or central nerve injury has been especially a hot topic for several decades. Though elongating and sprouting degenerated axons in the central nervous system (CNS) has some obstacles, the peripheral nervous system (PNS) is capable of regenerating its severed/amputated axons without any interventions. After nerve injuries, the proximal and distal stumps of the injured nerve undergo structural and molecular changes, in particular, in preparation for the process of axonal regeneration. During this process, the basement membrane which surrounds the axon and the Schwann cells in the PNS remain intact. Schwann cells line up in the basement membrane tube and synthesize growth factors, which attract axonal sprouts formed at the terminal of the proximal segment of the severed axon. The basement membrane tubes provide pathways for the regenerating axons to follow to muscles and skin. The Schwann cells then remyelinate the newly formed axons. Likewise, the oligodendrocytes perform the same function in the CNS. However, astrocytes in the CNS form a scar area after digesting the damaged axons, causing axons to have difficulties in regeneration toward the distal stump [1]. Despite of the capacity of the PNS to regenerate its damaged axons, regeneration has still limitations, resulting in the poor functional recovery.

Factors that are important for poor functional recovery are:

- invasion of macrophages cells into injured area and removal of the myelin and axon debris,¹
- slow regeneration of axons across the injury site,

¹This process is called Wallerian Degeneration

- muscle atrophy,
- axonal regeneration from the proximal stump into inappropriate distal parts,
- the distance from injury to the original target.

Thus, it can be said that the degree of functional recovery after an injury is dependent on the success in axonal regeneration, the accuracy of target reinnervation and the restitution of adequate connectivity in spinal circuits and central nervous system integration [2].

Many studies have shown that low-dose laser application meets these needs. It is only since the late 1980s that scientific interest were focused on this therapeutic approach for nerve regeneration and its usage has gained importance due to its non-invasiveness, application comfort through the skin surface and its effectiveness. Laser irradiation immediately after injury accelerates the rate of recovery of the injured nerve as shown by functional, morphological and electrophysiological evaluations [3]. However, there are few studies related to delayed use of the phototherapy [4, 5, 6]. Since pathologic changes occur at the first days, whether the laser slows the degeneration progress is unclear. This point needs to be clarified by further research. Therefore, there is a need to investigate the differences between starting times.

1.2 Objective

The objective of this thesis is to investigate whether there are functional, morphological and electrophysiological differences between the early and delayed use of laser phototherapy delivered to the crushed sciatic nerve.

1.3 Outline

The thesis is divided into four parts. In Chapter 2, the background information about the structure of peripheral nerves system, what changes are occurred in case of degeneration and how the natural mechanism reacts to deterioration and initiates the regenerative process. In Chapter 3, used experimental procedures are explained. In Chapter 4, results are presented and statistical differences are indicated. Finally in Chapter 5, an interpretation of the results along with a discussion of previous related studies are given.

2. BACKGROUND

Peripheral nervous system (PNS) is a network that connects central nervous system (CNS) to the body. PNS executes this function through the nerves that expand throughout the body. PNS is comprised of two parts: the somatic nervous system that receives sensory information from the sensory organs and regulates movements of the skeletal muscle and the autonomic nervous system the role of which is regulation of smooth muscle, cardiac muscle and glands. The somatic nerves are divided into two types depending on the place from which they are originated: spinal nerves and cranial nerves. Spinal nerves stem from the vertebral column whereas cranial ones emerge from the ventral surface of the brain. In contrast to spinal nerves, cranial nerves, except of the first two, namely the olfactory and optic nerves emerge directly from the ventral surface of the brain [1, 7].

In spite of the different roots of spinal and cranial nerves, both of them are branched by the sensory and motor nerves. Sensory nerves are responsible for relaying information from organs to brain and spinal cord that form CNS. On the other hand, PNS relays the response of CNS to muscles and organs through motor nerves. Thus, sensory nerves (incoming axons) are called as afferent axons while motor ones (outgoing axons) are referred to as efferent axons [1].

Peripheral nerve trunks that belong to spinal nerve originate from different locations. Motor nerves emerge from the ventral root (anterior root) of spinal cord whereas sensory neurons have their root from the dorsal root ganglion (spinal ganglion). At the distal part of each ganglion, sensory nerves join motor nerves forming spinal nerves [8].

Although peripheral nerve trunks have different roots and locate in the various part of the body, their morphology is almost similar with the only exception being the first two cranial nerves because they pertain to CNS [8].

2.1 Structure of Peripheral Nerve

The CNS has protection from mechanical injuries and toxins with the help of skull and blood-brain barrier. On the other hand, the PNS is protected by collagen sheath structure instead of skull and blood-brain barrier. This collagen sheath structure provides the PNS tensile strength and resistance to mechanical injuries. The collagen sheath covers the nerve from the inner to outer sides with layers, respectively endoneurium, Perineurium and epineurium. The nerve fiber that is the smallest functional unit of a peripheral nerve is enclosed by endoneurium; these nerve fibers are bundled up into groups called nerve fascicles, which are covered with perineurium; the outer layer is epineurium that covers groups of fascicles [7, 8].

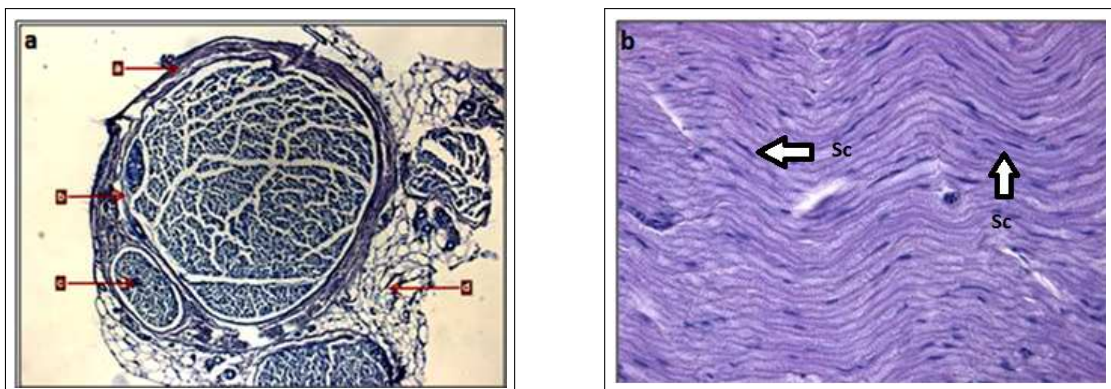


Figure 2.1 Illustration of intact peripheral nerve structure of Wistar rat. a) The cross section of sciatic nerve; a: epineurium, b: perineurium, c: fascicle, d: tissue structure. 10X magnification, HE stain b) The longitudinal section of sciatic nerve. 40X magnification, HE stain.

2.1.1 Endoneurium

The inner layer of the connective tissue is the endoneurium. It consists of axons, Schwann Cells (SCs), fibroblasts, macrophages, mast cells, collagen fibers and a capillary network. The endoneurium protects the nerve fiber with its endoneurial fluid which is a low protein fluid that is the peripheral nervous system equivalent of the cerebro-spinal fluid in the central nervous system. The pressure of the endoneurial fluid is slightly higher than that of the surrounding epineurium. In addition to the

endoneurial fluid, endoneurium has a matrix which is rich in regard to types I and III collagens. This gives it elasticity during movement and also protects the nerve against external trauma [7, 8].

2.1.2 Perineurium

The perineurium, the second layer of peripheral nerve, envelopes a group of nerve fibers called fascicle. The perineurium consists of collagen fibers and pinocytotic vesicles. Collagen fibers by which the perineurium cells are interconnected form a tight junction. On the other hand, pinocytotic vesicles within the perineurium are responsible for trans-cellular transport. Hence, the tight junctions between perineurial cells and pinocytotic vesicles within them form blood nerve barrier (BNB [8]. Like blood brain barrier in the CNS, the BNB controls the transmission of blood-borne structures to endoneurium, thus protecting the endoneurium from harmful constituents. In addition, maintaining osmotic milieu and the fluid pressure within the endoneurium is obtained thanks to perineurium with BNB [7, 8].

2.1.3 Epineurium

The epineurium that surrounds the multifascicular layer is the outer layer of peripheral nerve. As a general rule, the more fasciculi present in a peripheral nerve, the thicker the epineurium [7]. It contains collagen tissue sheath, some resident macrophages, fibroblast, and mast cells. One of the most important features of the epineurium is including the main supply channels of the intraneural vascular system: the vasa nervorum. Another equally important quality is having adipose tissue, providing epineurium with an important role in protection of nerve. Moreover, the epineurial sheath has little connection to adjacent tissues allowing nerves to normally slide and move during limb movement [8].

2.2 Nerve Types and Structure

Two major types of axons are identified within the endoneurium: myelinated and unmyelinated axon [7].

2.2.1 Myelinated Nerve Fiber

The production of layer composed of myelin around the axon is called myelination. Myelin is electrically insulating material that consists of water, proteins and mainly lipid. The cell responsible for myelination in the CNS is oligodendrocyte whereas myelin is synthesized by the Schwann cells in the PNS. It is not a general rule but it can be considerable that myelinated axons have diameters above 1.5 mm in the PNS. A myelinated nerve fiber, larger in caliber consists of an individual axon enveloped by a single Schwann cell [7]. The Schwann cell is not only responsible for myelination but also nerve development and regeneration, trophic support (nourishing) for neurons and production of the nerve extracellular matrix.

The membrane of this Schwann cell wraps around the nerve fiber to form a multilaminated myelin sheath. Myelination is critical for the conduction of impulses through axon. This is because Schwann cells forms Ranvier nodes between internodes in which the axon membrane is exposed to intracellular fluid containing sodium and potassium. Interaction of intracellular fluid allows the nerve impulses called action potential to be transmitted. This situation is coined as salutatory transmission [1, 8].

2.2.2 Unmyelinated Nerve Fiber

Unmyelinated nerve fibers are smaller in caliber between 0.15 and 2.00 mm in diameter. They are composed of several axons surrounded together by only one Schwann cell [7].

The speed of nerve impulses changes dramatically in respect to whether presence of myelin or not. The Table 2.1 illustrates different types of nerve fiber and their speed.

Table 2.1
The speed of myelinated and unmyelinated axons

Fiber Type	Myelination	Function	Diameter (μm)	CV(m/s)	
A	α (efferent)	+	Proprioception, somatomotor	12-20	100
	β (afferent)	+	Touch, pressure	5-12	30-70
	γ (efferent)	+	Motor to muscle spindle	3-6	15-30
	δ (afferent)	+	Pain esp. cold, touch	2-5	12-30
B		+	Preganglionic autonomic	< 3	3-15
C		-	Thermal pain, mechanoreceptor	0.4-1.2	0.5-2
		-	Postganglionic autonomic	0.3-1.3	0.7-2.3

2.2.3 Action Potential

The action potential (AP) is a phenomenon in which the electrical membrane potential of a nerve cell rapidly rises and falls because of special types of voltage-gated ion channels embedded in a cell's plasma membrane (Figure 2.2). On the other hand, the compound action potential (CAP) is the summation of all the action potentials produced by all fibers that were fired by the same stimulus [1].

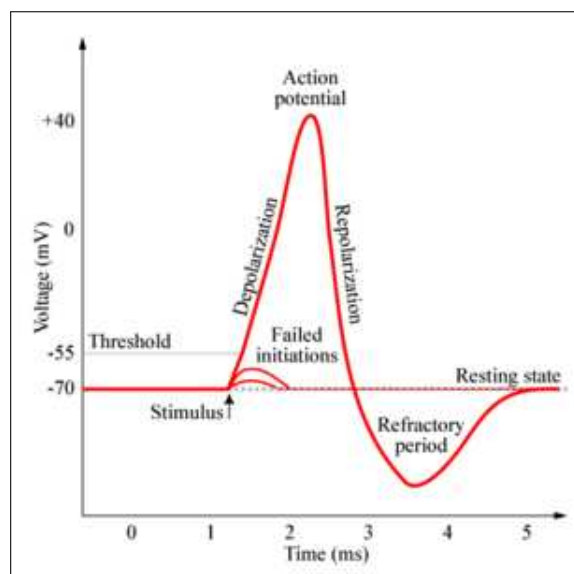


Figure 2.2 The Action Potential (AP)

The nerve is composed of many fibers with different diameters, and they are distributed at random throughout the nerve. The threshold for discharge of an axon in response to externally applied current is inversely related to the diameter of the axon, so that the large axon will have the lower threshold in case a large and small one is equidistant from the stimulating electrode. In addition, fibers contribute voltage to the compound potential in proportion to the square of their diameters. The result is that large fibers give the largest components to the compound action potential (Figure 2.3) [9, 10, 11].

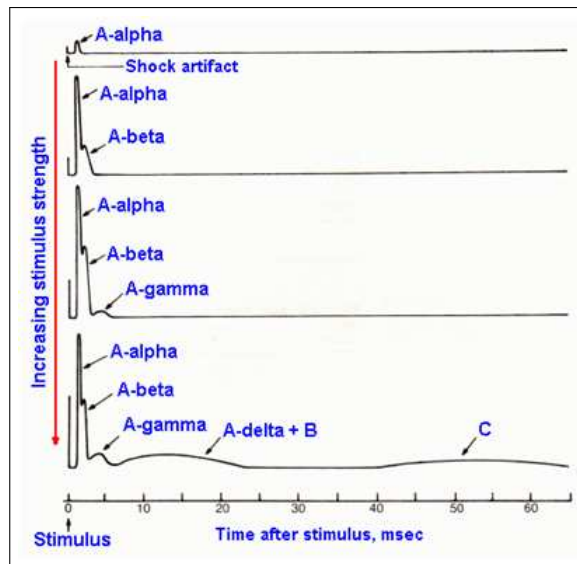


Figure 2.3 The speed of nerve fibers

2.2.4 Axon Ultrastructure

The intact mature peripheral nerve axon is constructed of a scaffold of proteins consisting of microfilaments, neurofilament intermediate proteins, and microtubules all surrounded by an axolemma. Microfilaments are expressed in the subaxolemmal cytoskeleton, whereas neurofilaments and microtubules are distributed throughout the axon [7].

2.3 Degeneration in PNS

There are, on the average, 50,000 cases of peripheral injuries recorded in America every year. Limited reported data are available to determine the incidence of peripheral nerve injuries. In North America, data taken from a trauma population in Canada revealed that approximately 2-3% of patients had a major nerve injury. In New South Wales, Australia, 2% of patients were reported to have a major nerve injury [12].

Peripheral injuries can be derived from compression, chemical toxins, transaction and trauma. Severity of injury is depended on location of damage in compartments of peripheral nerve [13]. The injuries firstly classified by Seddon are divided into neurapraxia (Class 1), axonotmesis (Class 2), and neurotmesis lesions (Class 3) regarding to where the damage occurred in the nerve.

Neurapraxia is the less severe injury in which there is only a lack of conduction as a result of demyelination. The axon and its connective tissue remain intact. There is no Wallerian degeneration and nerve is recovered completely.

In *axonotmesis*, axons of nerve are disrupted because of mechanical, chemical or ischemic lesion, yet its connective tissue are not. Due to the protection of connective tissue, the connection between the distal and the proximal stump of the nerve is maintained. After damage in axons, Wallerian degeneration mentioned below starts in the distal stump of the nerve to remove damaged tissue.

Neurotmesis is the most serious injury in which both axons and the connective tissue of the nerve are damaged. This type of injury is generally a result of transaction by which the proximal and distal stump is separated. Because of separation of distal stump from the nerve cell body, there is Wallerian degeneration in the distal part. Although the nerve is capable of regeneration after degeneration in axonotmesis, the only change for regeneration in neurotmesis is to connect the stumps of the nerve with suture or graft techniques. Despite surgical intervention, there is still a lack of recovery [8].

Addition to Seddon classification: Sunderland added 2 more groups into injury types related to damage in endoneurium and perineurium as summarized in Table 2.2 [8].

Table 2.2
Peripheral nerve injury classification done by Sunderland

Type 1	Conduction block (Neurapraxia)
Type 2	Unmyelination and Axonal Injury (Axonotmesis)
Type 3	Type 2 + Endoneurium Injury
Type 4	Type 3 + Perineurium Injury
Type 5	Type 4 + Epineurium Injury (Neurotmesis)

1. *First-degree injury* similar to neurapraxia mentioned above and is defined as an injury without structural interruption of the axon or subsequent Wallerian-like degeneration. The lesion is characterized by local demyelination.

2. *Second-degree injury* is similar to axonotmesis mentioned above. The injury does not disrupt the basal lamina or endoneurial integrity but the axon is damaged and there is distal Wallerian-like degeneration. Recovery occurs through regeneration of axons.

3. *Third-degree injury* involves a loss of axon continuity and subsequent Wallerian-like degeneration but the perineurial sheath remains intact. The general arrangement of the fascicles is preserved but there is endoneurial disruption.

4. *Fourth-degree injury* involves a severe disruption of the peripheral nerve trunk with disorganization of its topography and loss of fascicular arrangement. The nerve trunk is in continuity because the epineurium is not separated.

5. *Fifth-degree injury* involves a complete transection or separation of the entire nerve trunk, as described with the term neurotmesis [8, 14].

2.4 Regeneration in The PNS

After injury of PNS, axonotmesis or neurotmesis, similar morphologic and metabolic changes occur at injured nerve. These changes also occur in the nerve cell body, the proximal and distal part to injury site, the muscle end-plates and sensory receptor to which injured nerve is innervated. The proximal stump refers to the segment near the injury site that is connected to neuron cell body, whereas the distal stump means the other segment near to injury site that is attained to the end of axon. During degeneration and regeneration processes, several components of nerve change their regular function. Hence, neurons switch its role from relaying signal to growing mode, and Schwann cells responsible for myelination start to degrade myelin sheath. Also, protein syntheses are done to produce growth factor instead of the production of neurotransmitter [14].

The processes that take place after injury are divided into mainly three parts: Wallerian degeneration, axon regeneration/growth, and nerve reinnervation and that can be summarized as below:

1. *Excitability*: At initial stage of Wallerian degeneration signaling pathway in the injured axon works at normal rate and the distal stump of the damaged nerve tend to remain electrically excitable. However, the axon losses the ability of excitability which in turn slows down axonal transport at 2-3 days after damage [15].

2. *Chromatolysis*: At the neuron cell body, Nissl bodies that consist of arrays of rough endoplasmic reticulum and ribosome clusters break up and disperse. In addition, the nucleus migrates to the periphery of the neuron cell body. Disorganization of Nissl body called chromatolysis enables an increase in protein synthesis for axonal regeneration [14, 15].

3. *Cessation of interaction between the axon and the extracellular fluid:* Immediately after transaction of nerve, the open ends of axon are exposed to extracellular calcium and sodium ions causing an electrical signal that is conveyed to the nerve cell body. The voltage-dependent ion channels are opened and calcium ions flow into the axon. As a result of calcium influx, several proteinases responsible for breakdown of axonal structures (axolemma, neurofilament and neurotubules) are produced. Then, axon seals the plasmatic membrane at its edges ceasing interaction between the axon and the extracellular fluid.

4. *Wallerian degeneration:* During Wallerian degeneration, the Schwann cells degrade myelin sheaths of injured axon to remove inhibitory effect of myelin on axonal regeneration. The signal that triggers degradation of myelin results from the separation of neurolemma (the membrane of Sc) from axolemma (the membrane of axon).

5. *Phagocytosis:* It is not just Schwann cells that phagocytose myelin sheath and debris but also macrophages. Through the signalling of Schwann cells, macrophages invade injured nerve. It is surprising that they only accumulate around degenerating fibers not around intact nerve. This may be due to molecules which belong to chemokine family.

6. *Axon guidance:* Axons degenerate leaving the endoneurial tube behind in both the proximal and distal segment. However, the proliferation of Schwann cells fill in the endoneurial tube forming Bünger tube in the later segment. Bünger tube guides the growth cones in the proximal stump to reach the distal stump by secreting adhesion molecules. The Schwann cells also produce neutrophic factors that facilitate nourishment for axon cues.

7. *Proliferation of Schwann cells:* Proliferation of Schwann cells forms Bünger tube along endoneurial tube in the distal stump to the injury site. The loss of axon–Schwann cell contact generates a signal that causes Schwann cell proliferation. The Schwann cells upregulate the synthesis of several types of neurotropic factors such as NGF and BDNF. Proliferating Schwann cells organize themselves into columns and

the regenerating axons associate with them by growing distally between their basal membranes. During normal development, the Schwann cells proliferate to accommodate neurite elongation. This proliferation ceases during maturity and the Schwann cell population becomes quiescent. However, after nerve injury, the Schwann cells regain their proliferative phenotype and cell division starts at day 2 and peaks rapidly at day 3 post axotomy. At later time points examined (days 18 and 30) Schwann cell proliferation is markedly decreased.

8. *Growth cones in the proximal, capable of degrading cellular debris:* The growth cone has the ability to produce a protease that digests any material or debris that remains in its path of regeneration toward the distal site. The growth cone responds to molecules produced by Schwann cells such as laminin and fibronectin.

9. *Facilitation of axonal growth:* Molecules secreted by macrophages such as cytokines shifts SC functions from demyelinating to forming neurotrophins and adhesion molecules. Neurotrophins, NGF and CNF, facilitate axonal growth. On the other hand, adhesion molecules into Schwann cells of the distal stump attract growth cones.

10. *Pruning:* During regeneration, the excess number of cones reaches the distal stump. With time some of them that reach the appropriate target enlarge and mature. The others that do not reach the target are pruned and disappear. This process is called pruning.

11. *Myelination and compartmentation:* At the initial stage of regeneration, new axons are not enclosed by myelin sheath. Schwann cells produce myelin and envelope the axons forming a mature nerve fiber. After myelination, compartmentation (endoneurium, perineurium and epineurium) takes place completing the formation of the intact nerve [15] (Figure 2.4).

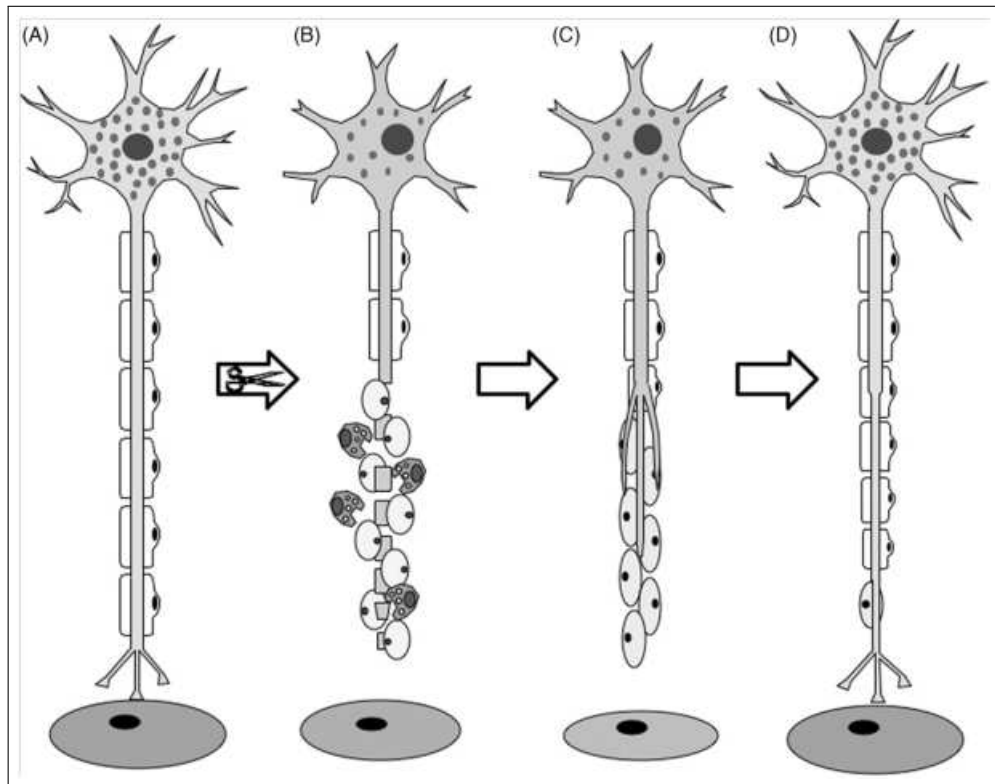


Figure 2.4 Schematic of the main events of degeneration and regeneration after peripheral nerve injury. (A) Normal nerve fiber, maintaining synaptic contact with target cells. (B) Transection of the fiber results in distal fragmentation of axon and myelin sheaths. Macrophages and Schwann cells phagocyte degraded materials. Chromatolysis at the neuron soma and dendritic arbor retraction occur. (C) Fine sprouts emerge from the proximal axonal end, and elongate in association with the proliferated Schwann cells in the distal segment, that line up in bands of Büngner. (D) Axonal reconnection with target cells and maturation of the nerve fiber. The regenerated axon remains of smaller caliber and with shorter internodes than normal. The neuron returns to a normal transmitting phenotype. Target cells may suffer atrophy and phenotypic changes during denervation.

Despite the ability of peripheral nerve to regenerate, the rate of axonal regeneration is initially very slow, and reaches a constant value by 3–4 days after injury, which is about 2–3 mm/day. This permanent impaired sensory and motor function results in neuropathic pain and major social consequences. To relieve this, many therapeutic intervention strategies for peripheral nerve repair such as delivering growth factors, electrical stimulation, physical activities and surgical repair have been tried. So far, phototherapy remains to be the most promising application and has been studied since the 80s, with numerous positive reports [16].

2.5 Laser in Repairing Injured Nerves

Laser is a term used for abbreviation of Laser Amplification by Stimulated Emission of Radiation. Laser creates and amplifies a narrow, intense beam of coherent light thanks to oscillator and population inversion [17].

Phototherapy is also named as low-power laser therapy due to low light radiation intensity. Energy intensity is so low so that the biological interaction of laser with cells and tissues is physical and chemical instead of heating [5]. The used energy spectrum is ranged from visible light to near-infrared light. The most commonly used wavelength is visible due many researches that report its beneficial effect for stimulating regenerative events.

Besides the wavelength, the treatment protocol can be modified by changing parameters such as the emission time, energy density, and the site of application. Although the most studies which used various laser parameters reported the positive effects of low-laser therapy, its mechanism is still poorly understood [3, 18].

3. MATERIAL AND METHODS

Thirty male adult Wistar rats weighing 291 ± 26 g in the same age line were obtained from the Life Sciences and Technologies Research Center of Bogazici University, Turkey. The experiments were done in physiology laboratory of Biomedical Engineering Institute. The animals were kept in plastic cages (three per cage) under controlled temperature (22 ± 2) with a 12 h light-dark cycle. The animals were fed rodent chow and water ad libitum. All procedures were approved by the Institutional Ethics Committee for the Local Use of Animals in Experiments (BÜHADYEK) under the protocol number 2011-10-25.

3.1 Surgical Procedure

The rats were weighed for calculation of the anaesthetic dose, and then they were anaesthetized with a mixture of ketamine (65 mg/1000 g body weight) and 2% xylazine (10 mg/1000 g body weight) with intra-peritoneal injection. After verifying the state of consciousness, each animal was positioned in the ventral decubitus position. The thigh of right hind leg of rat was shaved and sterilized locally by means of 5% alcohol and povidone iodide. An incision was made in the lateral face of this limb followed by a layer-by-layer dissection until the sciatic nerve was exposed. After separating the sciatic nerve from adjacent tissue, it was smashed from 1 cm proximal to its three main branches, called bifurcation (fibular, sural, and tibial) using the 5,000 g via dead-weight machine (DWN) developed by us used for 10 min as shown in Figure 3.1. During smashing period, the sciatic nerve was moisturized with saline in order to prevent it from drying.

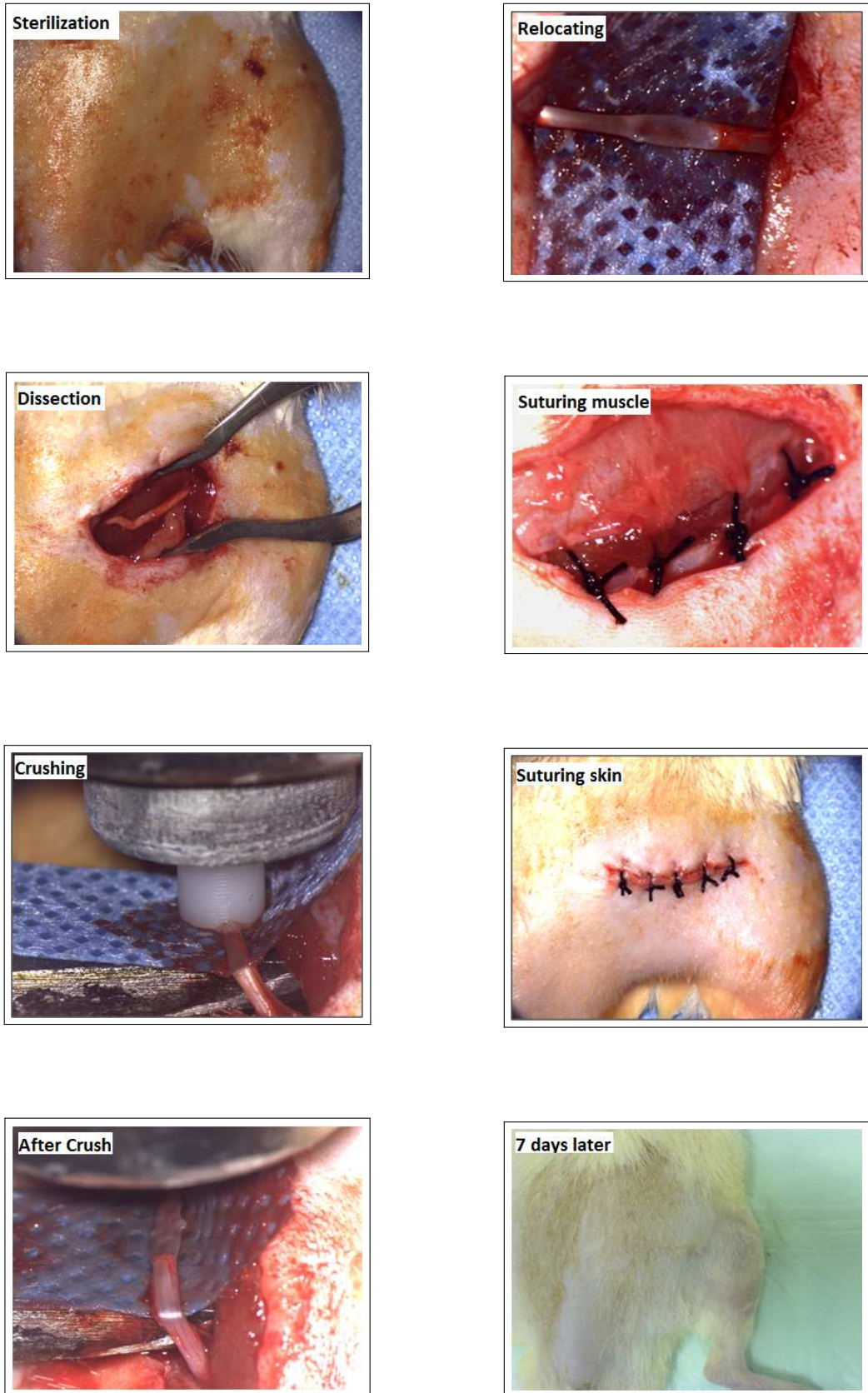


Figure 3.1 The surgical procedure

Then, the nerve was relocated in its bed, and the muscles and skin were sutured with simple stitches (Silk 3/0, Doğsan). After suturing, local asepsis was carried out, and each animal was given a dose of Gentamycin (10 mg/1000 g body weight) and Rimadiyl (5 mg/1000 g body weight) subcutaneously for prophylaxis of infections and promotion of analgesia.

The animals were divided into three experimental groups according to the procedure to be performed (n=10):

Table 3.1
Groups of animals used in present investigation

Experimental Groups (n=10)	Wavelength (nm)	Power (mW)	Energy Density (J/cm^2)	First Week	Second Week	Third Week
Control	-	-	-	-	-	-
Early Group	650	25	10	+	+	-
Delayed Group	650	25	10	-	+	+

Group 1 did not receive the laser treatment (untreated group),

Group 2 received the laser treatment starting on the first postoperative day and continuing for 14 days (early group),

Group 3 received the laser treatment starting 1 week after the injury and continuing for 14 days (delayed group).

Phototherapy

A 24 mW, 650 nm diode type laser with an approximate spot size of 0.14 cm^2 (Model: DH650-24-3(5), Huanic, China) was used in this experiment. The delivered energy density was chosen as 10 J/cm^2 in accordance with studies that reported success [19, 20, 21]. The average power of the equipment was measured as 25 mW before the experiment, with a power-measuring device (Low Power Detector, Model 918D-SL-0D3, Newport). Thus, the applied time was calculated as 56.7 sec to satisfy 10 J/cm^2 .



Figure 3.2 The measurement of laser power with a detector

Laser treatment was applied transcutaneously to the site of three points of surgical incision which was approximately 3 cm in all rats: one point at each extremity and another at the midpoint. The laser was positioned at an angle of 90° to the skin, according to the contact point technique. Prior to laser irradiation, the half dose of anaesthetic which found sufficient according to preliminary experiments was given to the rats. The therapeutic procedure was begun immediately after surgery in the early group and after 7 days in the delayed group. The therapy was continued for 2 weeks. All animals were treated the same way.

3.2 Functional Test: Sciatic Functional Index (SFI)

Initially, the animals were trained for two consecutive days on a walking track dimension of which is 42 x 8.2 cm as described by De Medinaceli et al. [22]. This track (Figure 3.3 (a)) was used to facilitate sampling of the hind footprints, which was necessary to calculate the Sciatic Functional Index (SFI). The footprints were obtained in the several time intervals that are the pre-operative period, and after seven, 14 and 21 post-operative days. The footprints were collected using stamp ink. The ink-soaked paws left footprints on strips of paper and three footprints per paw on average were recorded. The following formula proposed by Bain et al. (1989) was used to calculate the SFI. The distances between the second and fourth distal phalanges (IT), between the first and fifth distal phalanges (TS) and between the proximal edge of the foot and the third distal phalanx (PL) were measured (Figure 3.3 (b)). Negative scores

represent the percentage of functional loss [23].

$$SFI = -38 \cdot 3 \times \frac{(EPL-NPL)}{(NPL)} + 109 \cdot 5 \times \frac{(ETS-NTS)}{(NTS)} + 13 \cdot 3 \times \frac{(EIT-NIT)}{(NIT)} - 8 \cdot 8$$

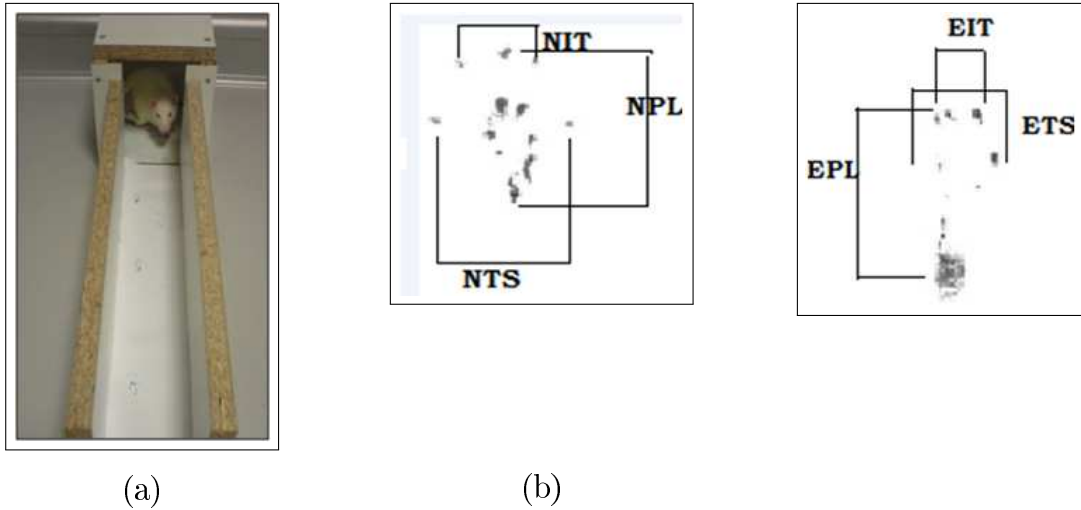


Figure 3.3 (a) Walking Track. (b) EPL, ETS and EIT stand for the experimental paw lengths, the experimental toe spread (distance between the 1st and 5th toe) and the experimental intermediary toe spread (distance between the 2nd and 4th toe), respectively. NPL, NTS and NIT stand for the normal paw length, normal toe spread and normal intermediary toe spread, respectively.

3.3 Electrophysiology

At the end of 21 postsurgery days, the rats were sacrificed with cervical dislocation. Both operated and intact legs were open and sciatic nerve was dissected. To record the conduction velocity (CV) and latency of compound action potential (CAP) of sciatic nerve invitro, stimulator and recording electrodes were used. Since the nerve gets dry easily after dissecting from its bed, it needs to be humisted. The Krebs Henseleit solution (112 mM NaCl, 4.6 mM KCl, 1.9 mM CaCl₂, 1.1 mM MgSO₄, 0.8 mM KH₂PO₄, 24 mM NaHCO₃, and 10 mM D-glucose adjusted to pH 7.2-7.4) is the best for moisturisation that is identical to body fluid of rat [24].

Using a Harvard Stimulator² a supramaximal square stimulus with a pulse-

²Harvard Nerve Stimulator

duration of 0.5 msec and 20V amplitude was delivered to the proximal segment of the sciatic nerve. The same stimulus was then applied to the distal segment of nerve. The replacement of distal and proximal stimulation is 0.5 cm. CAP was observed in the oscilloscope's screen (Tektronix TDS-1002b). Any appropriate response processed with a 5 kHz low pass filter was recorded. During recording, the sciatic nerve was moisturized with Krebs Henseleit solution.

Conduction velocity was calculated by dividing replacement (0.5 cm) to latency and the data obtained were recorded.

$$ConductionVelocity = \frac{(ProximalDistance - DistalDistance)}{(ProximalLatency - DistalLatency)}$$

3.4 Histology

The other method to observe recovery of crushed nerve is measuring the mononuclear cell in the crushed area. To count mononuclear cells, sciatic nerve was fixed in 10% formaldehyde buffer solution immediately after the CAP measurement. The fixation process is necessary to prevent postmortem decay including autolysis (self-digestion) and putrefaction (decomposition of protein structure of the cell).

The second part of tissue preparation for histology examination is dehydration. To cut tissue into thin slices, it must have strength and rigidity. Thus, water must be extracted from tissue and exchanged with a solidifying agent. Most common used solidifying agent is paraffin, but paraffin is immiscible with water. Thanks to dehydration process, water can be replaced with paraffin via transition agents which are alcohol replaced with water, xylene that removes alcohol and finally paraffin exchanged with alcohol. The dehydration process done after twenty-four hours fixation is summarized in Table 3.2 (a). Tissue processing was automatically performed by tissue processing machine (Leica TP-1020, Germany) in our Biophotonics Laboratory at the Institute of Biomedical Engineering, Bogazici University.

The third part of tissue processing is sectioning and staining. To section the sciatic nerves into thick slices, the processed tissue was embedded in blocks filled with paraffin. The orientation of tissue in paraffin blocks is very important. In this study, the nerves were aligned transversely in paraffin wax to observe the nerve fibers and the mononuclear cells between them. Firstly, the heated plate of embedding machine (Leica EG – 1150 H, Germany) was used to position the nerve segment in the blocks. Then, paraffin was cooled using cold plate. After enough time for cooling paraffin, the blocks were placed into microtome (Leica RM – 2255, Germany) capable of sectioning 10 μm thick tissue. 10 μm thick sections were placed on glass slides using 45 $^{\circ}\text{C}$ bath then put into incubator (Nüve EN-025) adjusted to 37 $^{\circ}\text{C}$ overnight for vaporization of water. The nerve slices were stained with Hematoxylin and Eosin which are the most frequently used stains in histology. Hematoxylin, a basic dye, stains the nucleus of the nerves blue; whereas eosin, an acidic dye, stains the cytoplasm of nerve fibers to pink.

Table 3.2

a) The steps of dehydration procedure

Dehydration Procedure		
Alcohol	70%	1 hour
Alcohol	80%	1 hour
Alcohol	80%	1 hour
Alcohol	96%	1 hour
Alcohol	96%	1 hour
Alcohol	100%	1 hour
Alcohol	100%	1 hour
Xylene		1 hour
Xylene		1 hour
Paraffin	60 $^{\circ}\text{C}$	1 hour
Paraffin	60 $^{\circ}\text{C}$	1 hour

b) Summary of H&E staining steps

H&E Staining		
Xylene		5 min
Alcohol	100%	10 dip
Alcohol	90%	10 dip
Alcohol	70%	10 dip
Water		10 dip
Hematoxylin		90 sec
Water	100%	10 dip
Alcohol	90%	10 dip
Alcohol	70%	10 dip
Eosin		2 min
Water		10 dip
Alcohol	70%	10 dip
Alcohol	50%	10 dip
Xylene		2 min

Ten images of each animal were captured using a Leica DM200 microscope and analyzed with Image J. Mononuclear cells representing inflammatory infiltrate were observed and the number of mononuclear cells was recorded in the fields equal to 10 4 μm^2 .

3.5 Statistical Analysis

Functional, morphometric and electrophysiological data were expressed as mean \pm SD. Functional data between groups were compared by the nonparametric Kruskal-Wallis test. Differences in SFI scores on different days were analyzed using Friedman test. Differences in nerve conduction velocity and latency of CAP and differences in the number of mononuclear cells were analyzed by One-Way ANOVA with Post-hoc Tukey.

Significance was set at $p < 0.05$.

4. RESULTS

4.1 Functional Test: Sciatic Functional Index (SFI)

To observe the progress and the functional recovery of animals, the *Sciatic Functional Index* was used. As expected, it was observed that all animals that underwent surgery could not use their affected foot effectively, causing walking problems. Walking ability deteriorated for the first and the second week in all the experimental groups. However, this condition improved and the animals started to walk normally, especially in the last week.

Table 4.1
Values obtained by Sciatic Functional Index mean \pm SD

Group	Pre-Surgery	7 days	14 days	21days
Control	- 8.4 \pm 12.67	- 72.45 \pm 5.75	-79.0 \pm 12.7	-55.46 \pm 18
Delayed	- 8.75 \pm 5.96	- 72.0 \pm 7.45	-75.3 \pm 6.96	-46.2 \pm 21.5
Early	- 6.65 \pm 9.73	- 70.2 \pm 3.54	-77.2 \pm 7.5	-51.1 \pm 19.4

The analysis of the SFI value obtained using Equation 3.2 is consistent with observation. Table 4.1 shows the data on the SFI during different experimental days. As expected, the SFI values of all groups in the pre-surgery are higher than those for the other days. According to Table 4.1, it can be concluded that the Early Group on the 7th day and the Delayed Group on the 14th and 21st days show better result in comparison with the other groups on these days. However, there are no significant differences between the groups at different days ($p > 0.05$). There are also no significant differences between pre-surgery values and the values on the 21st days after operation ($p > 0.05$, Figure 3.1) in all groups, but values on 7th and 14th days showed significantly differences compared to pre-surgery values and those on 21st days ($p < 0.05$).

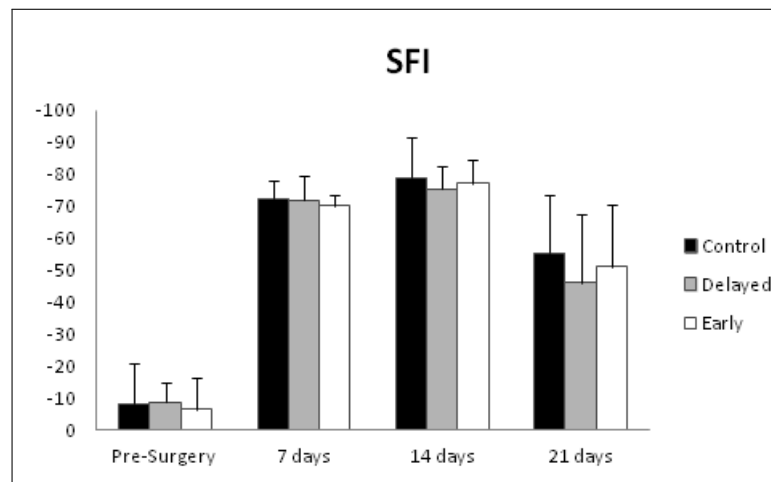


Figure 4.1 Mean and standard deviation values of the SFI obtained from three groups at pre-surgery, the 7th day, the 14th day and the 21th day.

4.2 Electrophysiology

Velocity and latency values of the compound action potential for all groups were recorded after 21 consecutive days. The values of contralateral (left) side were submitted to Normal groups. A total 29 data could be measured, leading to unequal sample size as indicated in Table 4.2.

Table 4.2
Velocity and Latency of Compound Action Potential

Group	Control(n=5)	Delayed(n=8)	Early(n=8)	Normal(n=8)
Latency(ms)	2.2±0.29	1.57±0.39	1.68±0.45	0.55±0.1
Velocity(m/s)	0.23±0.32	3.3±0.73	3.17±0.86	9.32±1.37

One-way ANOVA test showed that latency of all experimental groups is significantly different than the latency value of the Normal Group. In addition, the difference between the latency of the Delayed and Control Groups are significant ($p < 0.05$) based on post-hoc Tukey test (Figure 4.2 (a)). On the other hand, Figure 4.2 (b) illustrates that there is no significant difference between velocities of all groups ($p > 0.05$), but all of them are different compared to velocity of Normal group ($p < 0.05$).

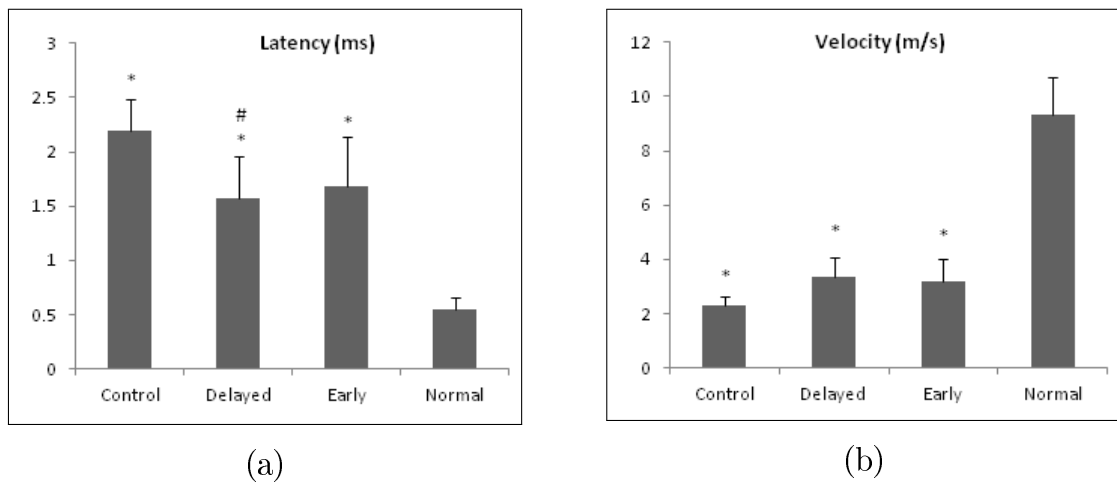


Figure 4.2 Contralateral sciatic nerves are submitted to Normal groups. a) Mean latency of compound action potential. All groups are significantly different than Normal group ($p < 0.05$). There is also a significant difference between Delayed and Control groups ($p < 0.05$). b) Mean velocity of compound action potential. No significant difference between groups except that velocity of normal group is significantly different than other groups ($p < 0.05$).

4.3 Histology

In addition to functional and electrophysiological tests, morphological examinations of the crushed nerves were performed. To indicate the possible recovery of sciatic nerve, the numbers of mononuclear cells were counted in the crush lesion segment divided to fields of areas equal to $10^4 \mu m^2$. The shape of the mononuclear cells in the lesion were round whereas the Schwann cells were interspersed among the nerve fibers and had fluted appearances (Figure 4.4). This feature was used for separating mononuclear cells from the Schwann cells and to count them manually. This counting technique called morphometric analysis showed that all the experimental groups have more infiltration than Normal groups ($p < 0.05$) due to higher number of mononuclear cells. However, groups in which laser was applied (Delayed: $12.62 \pm 2.81 \times 10^4 \mu m^2$ and Early: $13.38 \pm 2.85 \times 10^4 \mu m^2$) had less mononuclear cells compared to control group ($15.2 \pm 2.92 \times 10^4 \mu m^2$; $p < 0.05$). To exclude the effect of beginning time of laser application, analysis between Delayed and Early groups was also performed and no significant difference was found ($p < 0.05$) (Figure 4.3).

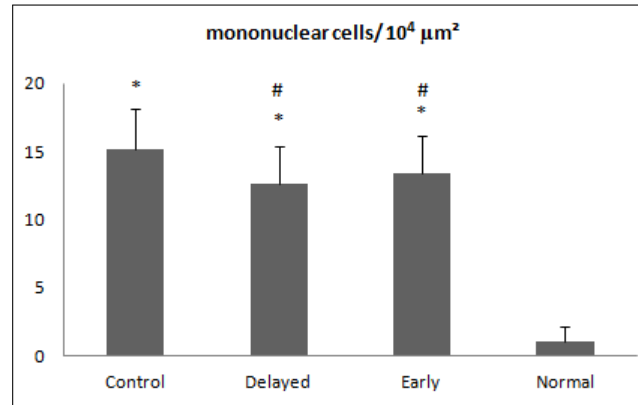


Figure 4.3 The number of mononuclear cells at the end of the study. All animals underwent crush lesion expect of Normal. No intervention in Control. Delayed and Early groups are submitted to laser phototherapy 7 days after surgery and immediately after surgery, respectively. The mean number of cells counted in images $10^4 \mu\text{m}^2$ captured at 400X. After 21 days, the number of mononuclear cells was significantly lower in both Delayed and Early groups than Control ($p < 0.05$, ANOVA and post hoc Tukey test). $p < 0.05$, in related to Normal group. $p < 0.05$, in related to Control group.

In addition to a decrease in the mononuclear cells in the laser groups, examination under light microscopy showed that both the Delayed and the Early groups got more dense fiber area, indicating myelination. Moreover, appearance of the samples that belong to the laser treated groups resembles the native nerve sample (Figure 4.4). This is another indication of nerve recovery.

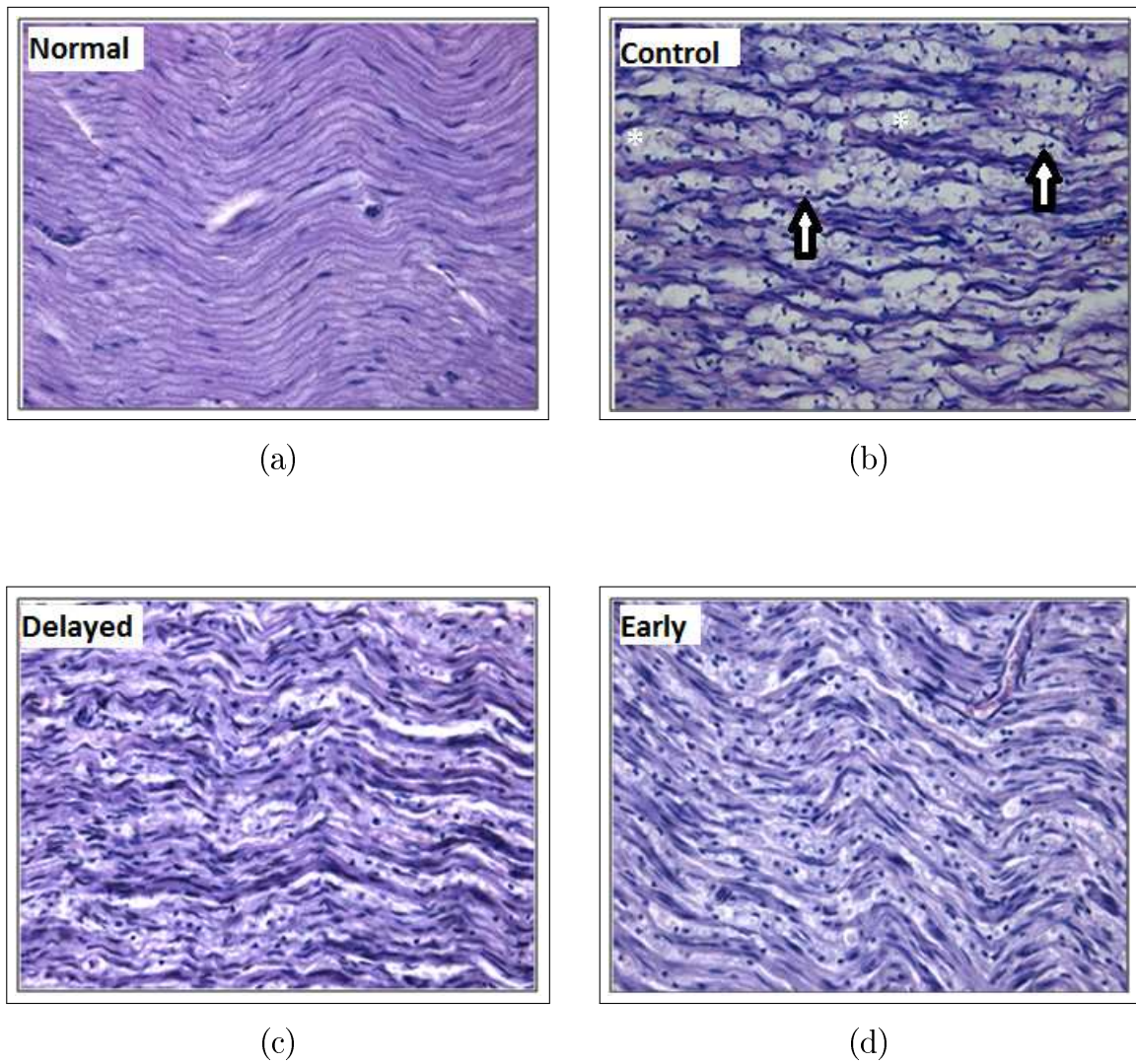


Figure 4.4 a) Intact nerve b) Sciatic nerve crush lesion without any treatment. An increase in the number of mononuclear cells (arrows), myelin degeneration (*) c) Sciatic nerve of delayed group d) Sciatic nerve of early group.

5. DISCUSSION

5.1 The Experimental Set Up

The objective of this study was to answer the question of whether effects of subsequent (early) and delayed laser radiation on crushed sciatic nerve regeneration shows differences. To observe the rate of recovery, evaluation was done by means of functional, electrophysiological and histological techniques.

Towards this objective, rats were chosen as subjects for experimentation because of their nerve anatomy similar to humans and easiness in their handling. Although reaching the sciatic nerve and determining a standard location for creating lesion seems to be difficult, the knowledge that this nerve is divided into three branches (tibial, fibular and sural nerve), provides the needed guidance. This branch point was taken as a reference position in creating the crush lesions for all the animals.

Although the crush type injury called axonotmesis is less severe than neurotmesis, it is mostly used in crush models since it enables experimenters to easily perform surgery who may not be proficient in micro-surgery. Moreover, the endoneurial tube is preserved after axonotmesis, giving the opportunity to observe the effect of any intervention on regeneration for a short time [13, 25].

To create the most used injury type, many researchers have preferred the use of standard surgical hemostatic clamps [13, 26, 27, 28]. Rockhind and his coworkers showed that the pressure of hemostat is 6.3 ± 0.7 MPa and its usage for 30 sec is sufficient to obtain crush models [29]. To overcome inhomogeneous crush area, special non-serrated hemostat tweezers were produced [19, 30]. To control, standardize and ensure duplicability of the lesions, however, we have preferred the use of another device instead of the hemostats. This is the so-called “dead-weight machine”. It has been shown that the dead weight machine with weights of 500, 1000 and 15.000 g provides

adequate crush lesions [31].

Mazzer et al. (2008) used 500, 1000, 5000, 10 000 and 15 000 g weights to smash nerve. They observed that 5.000 g weight applied for 10 minutes is enough and effective for the crush model since it caused sufficient deterioration in axon diameter, the number of fiber and myelinated area [32].

We also used dead-weight machine with 5.000 g equal to 50 N for 10 min that is more reliable application for axonotmesis [17].

Electrical, exercise and electro-acupuncture stimulation are used for the sake of nerve regeneration. Some of them induce successful recovery [33, 34, 35, 36]. Besides these alternative therapy techniques, the use of the laser has gained importance due to its noninvasiveness, application comfort through the skin surface and its effectiveness. Reports of works related to low-power laser therapy have appeared since the 80s.

The first studies of Helium Neon laser gained success in nerve regeneration and its positive effects have been more dominant as compared to the other wavelengths in the success studies. Anders et al. (1993) have studied various wavelengths, ranging from the ultraviolet to the infrared energy spectrum (361, 457, 514, 633, 720, and 1.060 nm). They investigated their effects on facial nerve regeneration by counting the motor neurons corresponding to the crushed nerve. Although all wavelengths have a certain degree of beneficial effect on regeneration as compared to non-irradiated group, the 633 nm was found to be the most effective for biostimulation [37]. Barbosa et al. (2010) also applied laser therapy at wavelengths at 660 nm and 830 nm to treat crush lesion for 21 days at the same energy density of 10 J/cm^2 . According to the SFI analyses, animals subjected to 660 nm irradiation showed better healing [20]. In addition to the works related to crush models, transaction of sciatic nerve called neurotmesis has also used to investigate the effect of laser therapy. With mean of neurotmesis model, 660 nm laser and 808 nm laser at 10 and 50 J/cm^2 were delivered to complete resection of the sciatic nerve. The laser application with 650 nm at both energy densities for 15 days showed better results in gait analysis [21].

Another laser parameter that affects the rate of recovery is the total energy density delivered. Previous studies have shown that red light with 10 J/cm^2 energy density causes an increase in the number of fibers, the myelin and axon diameters [20, 21, 37]. Also, Gigo-Benato et al. (2010) illustrated that 660 nm with 10 J/cm^2 and 60 J/cm^2 provides neuromuscular recovery after nerve crush [19]. Under the light of previous studies, 650 nm with 10 J/cm^2 energy density was chosen in this study.

At this point, the question whether to start laser therapy immediately after axonotmesis or whether it is more reasonable to wait for a week to deliver laser irradiation arises. Most of the studies have adopted a treatment protocol of laser application immediately after surgery and they obtained positive results compared to non-irradiated animals [13, 19, 20, 21, 25, 26, 27, 28, 38, 39]. However, fewer studies are interested in the onset of application that delayed the beginning of the treatment to 24 hours after surgery and the second postoperative week, obtaining null and negative results, respectively [5, 6]. Bagis (2003) crushed the sciatic nerve bilaterally and used the 904 nm pulsed laser at 24 hours after post-surgery. According to the data on compound muscle action potential and histological examination, they observed no differences between the control and the laser group [6]. On the other hand, Chen (2004) created 10-mm gap between proximal and the distal stumps of nerve sutured into rubber tubes to overcome the facility of Bunker Tube. In this respect, they searched whether low-power laser irradiation performed one week after micro-surgical repair could accelerate the rate of regeneration between the gaps. The histological and visual examination revealed that non-irradiated animals had more mature structure with a higher number of myelinated axons [6]. Despite these unexpected results, the 904 nm laser irradiation on crushed median nerves in mainly delayed group in which the onset of application started after 7 postoperative days, showed larger myelinated fibers than the control animals, resulting in better functional recovery [4]. In regard to this study, we have also preferred to use the most commonly used wavelength which is 650 nm.

5.2 The Effect of Laser on Inflammation

After any injury in the peripheral nervous system, natural defense mechanism becomes activated for recovery. The first reaction is the breakdown of injured nerve structures and its removal with help of resident or recruited macrophages. The myelin debris must be phagocytosed by macrophage in this degenerative period since the myelin debris is found as an inhibitor for myelination and axon sprouting [40]. In addition to produce appropriate area for regeneration, macrophage cells must also produce growth factors that induce proliferation of Schwann cells and fibroblasts [41]. Although the macrophage cells have a critical role for regeneration, their persistent presence causes neuroma and painful sensitivity. Thus, a decrease in the number of mononuclear cells is a crucial indicator of regeneration with regard to the progressive aspect. It is reported that 904 nm light-emitting diode radiation on crushed sciatic reduces the number of the inflammatory cells and areas of edema [26]. The changes in the number of inflammatory cells in our experiments are in good agreement with this result. The number of mononuclear cells is lower in both the delayed and the early groups, indicating that delayed laser application has the same effect with subsequent laser application after surgery. However, this decrement is not enough to be recovered totally in respect to the mononuclear cell population of the intact nerve. It should be mentioned that the intact nerve has fewer macrophage cells which are resident [7].

5.3 The Effect of Laser on Functional Recovery

Functional tests have various types depended on which kind of nerve is investigated. Gait analysis is one of functional tests and is based on the position of finger and heel. This test is a cheap and easy way in the use of gait analysis. Another advantage of SFI is giving the chance to follow the degree of recovery until animals are sacrificed [22, 23]. Thanks to this feature, it could be observed that the gait of all animals got worse after their nerves were injured during the two weeks following surgery. At day 21, this situation was changed and animals could walk more comfortably. Mean val-

ues of the SFI also justified this observation. The increase in the mean value for the delayed group at 14 and 21 days and early group at 21 days in the SFI, indicating recovery were promising. However, the Friedman test did not indicate any significant differences. Despite the SFI data gathered in this study, low-laser therapy on crushed or transected nerve immediately after surgery increased the SFI in the second week [25, 26, 27]. The possible reason for the failure in the functional test may be inaccuracy in measuring the distance. As mentioned by Monte-Raso et. al., the value of the SFI measured by different experimenters can show variations [42]. A walking track equipped with a camera has been proposed to overcome this problem, thereby making dynamic evaluations possible [43, 44].

5.4 The Effect of Laser on CAP Response

It has been reported that 632.8 nm He-Ne laser radiation applied on the intact sciatic nerve, at an energy density between 4 J/cm^2 and 10 J/cm^2 delivered transcutaneously causes significant increase in action potential (AP) of irradiated nerves. It has also been reported that daily irradiation for 20 consecutive days showed that the cumulative effect of the He-Ne irradiation lasted nearly 8 months and the maximum AP was obtained at the 20th day [45].

5.5 Possible Mechanism of Laser Effects

Although the effects of low-energy laser are controversial, and its mechanism is not completely understood, some studies have tried to explain this phenomenon. It has been demonstrated that red light interacts with the chromophore of mitochondria, leading to acceleration in the respiratory chain and an increase in ATP synthesis [46]. Another attempt to show a possible mechanism for biostimulation was carried out by Shen et al (2003) who showed that irradiation with 650 nm laser for four consecutive days elevates the growth-associated protein-43 (GAP-43) in the crushed sciatic nerve.

As the level of GAP-43 is associated with the number of nerve sprouts, the rate of its expression can be indicative of nerve regeneration [27]. The injury in the PNS does not only initiate degenerative changes in the injured segment but also in the motor neurons of the corresponding segment of the spinal cord and in the muscle to which the injured nerve innervates. However, laser treatment of the crushed peripheral nerve stops pathologic changes and initiates proliferation of astrocytes and oligodendrocytes responsible for myelination [28]. In addition, it has been shown that red light with low energy density accelerates muscle recovery after injury to the sciatic nerve [19]. These findings clearly support the success of laser therapy in nerve regeneration.

Rochkind et al. (2007) is a pioneer of the application of low-power laser therapy in patients with long-term peripheral injury. In this pilot study, 780 nm laser at an energy density of 300 J/cm^2 was delivered to the injured peripheral nerve and to the corresponding segments of spinal cord for 21 consecutive days. The patients were monitored at the end of 21 days of treatment and after 3 and 6 months. The analysis of motor function and electrophysiological evaluation showed significant improvement in laser irradiated patients compared to the placebo group [47]. Although we have not performed any research on humans, our results on rats are encouraging and show that laser therapy can be effective in the treatment of peripheral nerve injuries.

5.6 Recommendations for Future Work

Based on the morphological and electrophysiological evaluations of the data presented here supports that delayed laser use could enhance nerve regeneration in. Thus, the future studies can be focused on the time set and onset of therapy.

To get further information about how delayed laser use effects on the nerve regeneration, the histological examination under which diameter of axon and myelin are measured can be done. This evaluation technique gives information about myelination a major factor in signal transmission and can be correlated with the amplitude and velocity of action potential of nerve [48].

In addition, the effects of the longer wavelength can be researched due to its high penetration depth that can be applied to patients suffering from peripheral nerve injuries.

REFERENCES

1. Carlson, N., *Physiology of behavior* ., Allyn & Bacon, 2010.
2. Deumens, R., A. Bozkurt, M. Meek, M. Marcus, E. Joosten, J. Weis, and G. Brook, “Repairing injured peripheral nerves: bridging the gap,” *Progress in neurobiology*, Vol. 92, no. 3, pp. 245–276, 2010.
3. Gigo-Benato, D., S. Geuna, and S. Rochkind, “Phototherapy for enhancing peripheral nerve repair: a review of the literature,” *Muscle & nerve*, Vol. 31, no. 6, pp. 694–701, 2005.
4. Santos, A., C. Suaid, M. Xavier, and F. Yamane, “Functional and morphometric differences between the early and delayed use of phototherapy in crushed median nerves of rats,” *Lasers in medical science*, Vol. 27, no. 2, pp. 479–486, 2012.
5. Bagis, S., U. Comelekoglu, B. Coskun, A. Milcan, B. Buyukakilli, G. Sahin, S. Ozisik, and C. Erdogan, “No effect of ga-as (904 nm) laser irradiation on the intact skin of the injured rat sciatic nerve,” *Lasers in medical science*, Vol. 18, no. 2, pp. 83–88, 2003.
6. Chen, Y., S. Hsu, C. Chiu, J. Lin, C. Chen, and C. Yao, “Effect of low-power pulsed laser on peripheral nerve regeneration in rats,” *Microsurgery*, Vol. 25, no. 1, pp. 83–89, 2005.
7. Geuna, S., S. Raimondo, G. Ronchi, F. Di Scipio, P. Tos, K. Czaja, M. Fornaro, *et al.*, “Histology of the peripheral nerve and changes occurring during nerve regeneration,” *Int. Rev. Neurobiol.*, Vol. 87, pp. 27–46, 2009.
8. Zochodne, D., “Neurobiology of peripheral nerve regeneration,” *Recherche*, Vol. 67, p. 02, 2008.
9. Navarro, X., and E. Udina, “Methods and protocols in peripheral nerve regeneration experimental research: part iii–electrophysiological evaluation.,” *Int Rev Neurobiol.*, Vol. 87, pp. 105–126, 2009.

10. Werdin, F., H. Grüssinger, P. Jaminet, A. Kraus, T. Manoli, T. Danker, E. Guenther, M. Haerlec, H. Schaller, and N. Sinis, “An improved electrophysiological method to study peripheral nerve regeneration in rats,” *Journal of neuroscience methods*, Vol. 182, no. 1, pp. 71–77, 2009.
11. Güclü, B., “Biomedical instrumentation laboratory manuals,” *Bogazici University Press*, 2006.
12. Stanfield, C., and W. Germann, “Principles of human physiology,” *Recherche*, Vol. 67, p. 02, 2010.
13. Belchior, A., F. dos Reis, R. Nicolau, I. Silva, D. Perreira, and P. de Carvalho, “Influence of laser (660 nm) on functional recovery of the sciatic nerve in rats following crushing lesion,” *Lasers in medical science*, Vol. 24, no. 6, pp. 893–899, 2009.
14. Navarro, X., M. Vivó, A. Valero-Cabré, *et al.*, “Neural plasticity after peripheral nerve injury and regeneration,” *Progress in neurobiology*, Vol. 82, no. 4, p. 163, 2007.
15. Norman, R., and M. Katarzyna, *Degeneration and Regeneration in the Nervous System.*, Harwood Academic, 2000.
16. Hamid, S., and R. Hayek, “Role of electrical stimulation for rehabilitation and regeneration after spinal cord injury: an overview,” *European Spine Journal*, Vol. 17, no. 9, pp. 1256–1269, 2008.
17. Breck, J., and E. J.H., *Introduction to laser technology.*, New York, 2001.
18. Rochkind, S., “Phototherapy in peripheral nerve regeneration: From basic science to clinical study,” *Neurosurgical Focus*, Vol. 26, no. 2, p. 8, 2009.
19. Gigo-Benato, D., T. Russo, E. Tanaka, L. Assis, T. Salvini, and N. Parizotto, “Effects of 660 and 780 nm low-level laser therapy on neuromuscular recovery after crush injury in rat sciatic nerve,” *Lasers in surgery and medicine*, Vol. 42, no. 9, pp. 673–682, 2010.

20. Barbosa, R., A. Marcolino, R. de Jesus Guirro, N. Mazzer, C. Barbieri, and M. de Cássia Registro Fonseca, "Comparative effects of wavelengths of low-power laser in regeneration of sciatic nerve in rats following crushing lesion," *Lasers in medical science*, Vol. 25, no. 3, pp. 423–430, 2010.
21. Medalha, C., G. Di Gangi, C. Barbosa, M. Fernandes, O. Aguiar, F. Faloppa, V. Leite, and A. Renno, "Low-level laser therapy improves repair following complete resection of the sciatic nerve," *Lasers Med Sci*, Vol. 27, pp. 629–635, May 2012.
22. de Medinaceli, L., W. Freed, and R. Wyatt, "An index of the functional condition of rat sciatic nerve based on measurements made from walking tracks," *Experimental neurology*, Vol. 77, no. 3, pp. 634–643, 1982.
23. Bain, J., S. Mackinnon, and D. Hunter, "Functional evaluation of complete sciatic, peroneal, and posterior tibial nerve lesions in the rat," *Plastic and reconstructive surgery*, Vol. 83, no. 1, pp. 129–136, 1989.
24. Pawson, L., L. Prestia, G. Mahoney, B. Güçlü, P. Cox, and A. Pack, "Gabaergic/glutamatergic–glial/neuronal interaction contributes to rapid adaptation in pacinian corpuscles," *The Journal of Neuroscience*, Vol. 29, no. 9, pp. 2695–2705, 2009.
25. dos Reis, F., A. Belchior, P. de Carvalho, B. da Silva, D. Pereira, I. Silva, and R. Nicolau, "Effect of laser therapy (660 nm) on recovery of the sciatic nerve in rats after injury through neurotmesis followed by epineural anastomosis," *Lasers in medical science*, Vol. 24, no. 5, pp. 741–747, 2009.
26. Serafim, K., S. Ramos, F. de Lima, M. Carandina, O. Ferrari, I. Dias, D. Togninho Filho, and C. Siqueira, "Effects of 940 nm light-emitting diode (led) on sciatic nerve regeneration in rats," *Lasers in medical science*, Vol. 27, no. 1, pp. 113–119, 2012.
27. Shin, D., E. Lee, J. Hyun, S. Lee, Y. Chang, J. Kim, Y. Choi, and B. Kwon, "Growth-associated protein-43 is elevated in the injured rat sciatic nerve after low power laser irradiation," *Neuroscience letters*, Vol. 344, no. 2, pp. 71–74, 2003.

28. Rochkind, S., I. Vogler, and L. Barr-Nea, "Spinal cord response to laser treatment of injured peripheral nerve," *Spine*, Vol. 15, no. 1, pp. 6–10, 1990.
29. Nissan, M., S. Rochkind, and M. Ringel, "Strain-gauged haemostatic forceps for clinical and experimental use," *Medical and Biological Engineering and Computing*, Vol. 26, no. 4, pp. 448–450, 1988.
30. Varejão, A., A. Cabrita, M. Meek, J. Bulas-Cruz, P. Melo-Pinto, S. Raimondo, S. Geuna, and M. Giacobini-Robecchi, "Functional and morphological assessment of a standardized rat sciatic nerve crush injury with a non-serrated clamp," *Journal of neurotrauma*, Vol. 21, no. 11, pp. 1652–1670, 2004.
31. Chen, L., A. Seaber, R. Glisson, H. Davies, G. Murrell, D. Anthony, and J. Urbaniak, "The functional recovery of peripheral nerves following defined acute crush injuries," *Journal of orthopaedic research*, Vol. 10, no. 5, pp. 657–664, 2005.
32. Mazzer, P., C. Barbieri, N. Mazzer, and V. Fazan, "Morphologic and morphometric evaluation of experimental acute crush injuries of the sciatic nerve of rats," *Journal of neuroscience methods*, Vol. 173, no. 2, pp. 249–258, 2008.
33. Brushart, T., R. Jari, V. Verge, C. Rohde, and T. Gordon, "Electrical stimulation restores the specificity of sensory axon regeneration," *Experimental neurology*, Vol. 194, no. 1, pp. 221–229, 2005.
34. Al-Majed, A., C. Neumann, T. Brushart, and T. Gordon, "Brief electrical stimulation promotes the speed and accuracy of motor axonal regeneration," *The Journal of neuroscience*, Vol. 20, no. 7, pp. 2602–2608, 2000.
35. Asensio-Pinilla, E., E. Udina, J. Jaramillo, and X. Navarro, "Electrical stimulation combined with exercise increase axonal regeneration after peripheral nerve injury," *Experimental neurology*, Vol. 219, no. 1, pp. 258–265, 2009.
36. La, J., S. Jalali, and S. Shami, "Morphological studies on crushed sciatic nerve of rabbits with electroacupuncture or diclofenac sodium treatment," *The American journal of Chinese medicine*, Vol. 33, no. 04, pp. 663–669, 2005.

37. Anders, J., R. Borke, S. Woolery, and W. Van De Merwe, "Low power laser irradiation alters the rate of regeneration of the rat facial nerve," *Lasers in surgery and medicine*, Vol. 13, no. 1, pp. 72–82, 2005.
38. Shamir, M., S. Rochkind, J. Sandbank, and M. Alon, "Double-blind randomized study evaluating regeneration of the rat transected sciatic nerve after suturing and postoperative low-power laser treatment," *Journal of reconstructive microsurgery*, Vol. 17, no. 2, pp. 133–138, 2001.
39. Mohammed, I., and L. Kaka, "Promotion of regenerative processes in injured peripheral nerve induced by low-level laser therapy," *Photomedicine and Laser Surgery*, Vol. 25, no. 2, pp. 107–111, 2007.
40. Stoll, G., S. Jander, and R. Myers, "Degeneration and regeneration of the peripheral nervous system: from augustus waller's observations to neuroinflammation," *Journal of the Peripheral Nervous System*, Vol. 7, no. 1, pp. 13–27, 2008.
41. Sanders, V., and K. Jones, "Role of immunity in recovery from a peripheral nerve injury," *Journal of Neuroimmune Pharmacology*, Vol. 1, no. 1, pp. 11–19, 2006.
42. Monte-Raso, V., C. Barbieri, N. Mazzer, A. Yamasita, and G. Barbieri, "Is the sciatic functional index always reliable and reproducible?," *Journal of neuroscience methods*, Vol. 170, no. 2, pp. 255–261, 2008.
43. Bervar, M., "Video analysis of standing—an alternative footprint analysis to assess functional loss following injury to the rat sciatic nerve," *Journal of neuroscience methods*, Vol. 102, no. 2, pp. 109–116, 2000.
44. Bozkurt, A., S. Tholl, S. Wehner, J. Tank, M. Cortese, R. Deumens, F. Lassner, F. Schügner, A. Gröger, R. Smeets, *et al.*, "Evaluation of functional nerve recovery with visual-ssi—a novel computerized approach for the assessment of the static sciatic index (ssi)," *Journal of neuroscience methods*, Vol. 170, no. 1, pp. 117–122, 2008.
45. Nissan, M., S. Rochkind, N. Razon, and A. Bartal, "Hene laser irradiation delivered transcutaneously: its effect on the sciatic nerve of rats," *Lasers in surgery and medicine*, Vol. 6, no. 5, pp. 435–438, 2005.

46. Rochkind, S., V. Drory, M. Alon, M. Nissan, and G. Ouaknine, "Laser phototherapy (780 nm), a new modality in treatment of long-term incomplete peripheral nerve injury: a randomized double-blind placebo-controlled study," 2007.
47. Wolthers, M., M. Moldovan, T. Binderup, H. Schmalbruch, and C. Krarup, "Comparative electrophysiological, functional, and histological studies of nerve lesions in rats," *Microsurgery*, Vol. 25, no. 6, pp. 508–519, 2005.
48. Martins, R., M. Siqueira, C. Silva, and J. Plese, "Correlation between parameters of electrophysiological, histomorphometric and sciatic functional index evaluations after rat sciatic nerve repair," *Arquivos de neuro-psiquiatria*, Vol. 64, no. 3B, pp. 750–756, 2006.