Çukurova Üniversitesi Tıp Fakültesi Dergisi
Journal of Cukurova University Faculty of Medicine

Araştırma Makalesi / Research Article

An Investigation of Correlation Between Electrophysiological and Functional Recovery After the Sciatic Nerve Injury

Siyatik Sinir Ezilmesinden Sonra Elektrofizyolojik ve Fonksiyonel İyileşme İlişkisinin Araştırılması

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Çukurova Üniversitesi Tıp Fakültesi Dergisi (Journal of Cukurova University Faculty of Medicine) 2012; 37(4):177-185.

ABSTRACT

Purpose: Video or photo assisted footprint analysis method is used to determine the motor and sensorial development instead of classic walking track footprint analysis in experimental peripheral nerve injury. Besides, the sucrose-gap method is used for measuring the electrophysiological activity in the sciatic nerves in-vitro. The aim of this study is to investigate the relationship between functional and electrophysiological recovery during the nerve regeneration in Wistar rats.

Methods: In the experiments, after the unilateral sciatic nerve crushing, the rats were evaluated at the preoperative and 2nd, 4th, 6th and 8th weeks postoperative using the sucrose gap method, and photo assisted footprint method. The compound action potentials (CAP), the Peak- time (PT) and the ½ Falling- time (1/2FT) were measured, and compared to functional results.

Results: Two weeks after being crushed sciatic nerves, complete function loss was seen operated legs in all rats. The amplitude of CAP was determined too small. The PT and the 1/2FT values were three fold longer than intact. However, following 4th – 8th weeks, the amplitude of CAP and other parameters of CAP were closed to intact values.

Conclusion: The findings indicated that the results of the functional recovery were correlated to electrophysiological results. However, functional results showed almost full functional recovery in the 4th week, the electrophysiological results did not reach to intact values in the 8th week. We conclude that photo assisted footprint analysis method and sucrose-gap technique, which are useful functional and electrophysiological methods to produce complementary knowledge with each other in the investigation of experimental peripheral nerve regeneration.

Key Words: Footprint, compound action potential, sucrose-gap, crush, regeneration, sciatic nerve

ÖZET

Amaç: Deneysel periferik sinir yaralanmalarında motor ve duyusal iyileşmeyi belirlemek için klasik yürüme izi analiz yöntemi yerine, video ya da fotoğraf destekli footprint analiz yöntemi kullanılabilmektedir. Bunun yanında in-vitro bir yöntem olan sukroz-gap yöntemi de sıçan siyatik sinirlerinde elektrofizyolojik aktivitenin ölçülmesinde kullanılmaktadır. Bu çalışmadaki amacımız Wistar türü sıçanlarda, sinir rejenerasyonu sırasındaki fonksiyonel ve elektrofizyolojik iyileşme ilişkisini araştırmaktır.

Yöntem: Çalışmalarımızda, siyatik sinirlerde tek taraflı ezildikten sonra, sıçanlar operasyon öncesi, 2., 4., 6. ve 8. haftalarda sukroz-gap ve fotoğraf destekli footprint yöntemi ile değerlendirildi. Bileşik aksiyon potansiyelleri (CAP), yükselme süresi (PT) ve ½ düşme süresi ölçüldü (1/2 FT) ve fonksiyonel sonuçlarla karşılaştırıldı.

Bulgular: Siyatik sinirlerin ezilmesinden iki hafta sonra tüm sıçanların ezilmiş sinirin bulunduğu ayaklarda tam fonksiyon kaybı oldu. CAP genliği çok düşük olarak belirlendi. PT ve 1/2FT değerleri normal değerlerinden üç kere daha uzundu. Buna rağmen, takip eden 4. - 8. haftalarda, CAP genliği ve diğer CAP parametreleri normale yakın değerlere geri döndü.

Sonuç: Bulgular, fotoğraf destekli footprint yöntemi sonuçları ile, elektrofizyolojik sonuçların yakın bağlantı içinde olduğuna işaret etmektedir. Bununla birlikte, fonksiyonel sonuçlar, 4. haftada neredeyse tam iyileşmeyi gösterirken, elektrofizyolojik ölçümler 8. haftaya kadar normal değerlere ulaşmadı. Sonuç olarak, fotoğraf destekli footprint analiz ve sukroz-gap yönteminin deneysel periferik sinir rejenerasyonun araştırılmasında birbirini tamamlayıcı bilgiler ürettiğini düşünüyoruz.

Anahtar Kelimeler: Footprint, Bileşik sinir aksiyon potansiyeli, Sukroz-gap, Sinir rejenerasyonu, Siyatik sinir

INTRODUCTION

The rat sciatic nerve crush injury is a well-established animal model and widely used in experimental studies for the study of regeneration of peripheral injuries. If peripheral nerves were damaged by the crush, the axons and their myelin sheaths degenerated with the distal stump called Wallerian degeneration[1-3]. Then, nerve fibers regrow to their targets at a rate of 3-4 mm/day[1,2]. During the Wallerian degeneration, abnormal electrophysiological properties appeared due to demyelination and other conditions [4-8]. Different techniques have been used to assess nerve recovery following peripheral nerve injury such as histological, electrophysiological and functional methods.

One of the functional methods is the footprint analysis which is useful, non-invasive method of measuring functional recovery after sciatic nerve injury and it shows a combination of motor and sensory recovery[9,10]. The footprint analysis uses walking pattern of the rat based on its footprint. This method generally used by the groups that work in the field of sciatic nerve regeneration[9-11]. Since its first description by De Medicaneli et al., it has been modified several times including video techniques[12,13]. We have used video based modification technique called video assisted footprint analysis technique described by Bervar, 2000[10].

The sucrose-gap which is an in-vitro method was used for measuring the electrophysiological activity of the nerve fibers such as compound membrane potential or compound action potential[14]. With this method, action potentials of all the fibers in the nerve bundle can be measured as a compound and its electrophysiological responses against different stimulation frequencies or pharmacological agents can be evaluated. The sucrose-gap technique is used by many researchers to investigate the regeneration in the crushed peripheral nerves[15-17].

While the video assisted footprint analysis method gives us information about the regenerating sensory and motor nerves that reach to the muscle, the SG method reflects the electrophysiological response of the regeneration in the crushed region. In the present scientific literature, although there are studies in which video assisted footprint analysis and sucrose-gap methods were separately used in the regeneration in the rat peripheral nerves, no study that uses both methods could be found. In our study, it was aimed to evaluate functional and electrophysiological development using together video assisted footprint analysis and sucrose-gap methods in the crushed rat sciatic nerves. While in the video assisted footprint analysis technique, which is noninvasive method, data can be obtained from the same experimental group in the different time intervals, in the SG method this can not be performed due to the necessity of the removal of the nerve bundle. So, in the animal groups, video assisted footprint analysis was performed at the outset, and then sucrose-gap method was used after removing of the sciatic nerves.

MATERIAL and METHODS

Animals and surgical operation

Adult female Wistar rats, between 230 and 260 g, were used for this study (n=50). The animals obtained from Cukurova University Medical Sciences Research Center. For this study was approved by ethic committee of the same center. The rats were distributed to 5 groups (intact, 2nd, 4th, 6th and 8th weeks) with 10 rats in each group. Photo assisted footprint recording of the rats in all the groups were performed at the outset, and then the sciatic nerves were removed and electrophysiological recordings were conducted using the sucrose-gap technique. All the animals were housed under temperature and light-controlled conditions (12:12 h light-dark cycle, 22 ± 2 ºC), with food and water available ad

libitum. Surgical procedures were performed using aseptic techniques and intraperitoneal deep anesthesia (Ketamine 8 mg/100 g, Xylasine 1 mg/100 g). Under deep anesthesia, the right sciatic nerve was exposed through a biceps muscle splitting incision in the posterior thigh. After nerve mobilization, the right sciatic nerves of rats were crushed 10 mm distal to the sciatic notch. The crushing of the nerve was performed by locking haemostatic artery fine forceps (Aesculab66) at its maximum point for 30s. After the skin were stitched with 3/0 prolene thread.

Electrophysiological experiments

The sucrose-gap technique was used to obtain a monophasic compound action potential (CAP) extracellulary[14]. The modified experimental set-up has been described previously[18-20]. Briefly, the sucrose-gap apparatus which having four pools; stimulating pool contains a pair of platinum electrodes, is filled with normal Krebs' solution; test pool contains test solution; sucrose pool contains isotonic sucrose and KCl pool contains isotonic KCl solution (Fig. 1A.). Pools are isolated from each other with vaseline-silicon oil mixture. The potential difference between test pool and KCl pool was recorded by using agar bridge Ag-AgCl electrodes. Before starting the experiment, the nerve was superfused with oxygenated Krebs' solution to achieve stable baseline and to record reproducible CAP. Following a period of stabilization (30-45 min), the nerves were placed in a sucrose-gap apparatus for stimulation and recording and it superfused with the appropriate solutions at a flow rate of 1-2 ml/min.

The rats were sacrificed in 2^{nd} , 4^{th} , 6^{th} and 8^{th} weeks by cervical dislocation, then their sciatic nerves were rapidly removed and desheated in Krebs' solution. Later, the crushed site was placed to the sucrose-gap apparatus with adjusting the drug compartment. During the experiments, nerves were stimulated supramaximally with 0.05 ms duration square-wave pulses. The nerve was stimulated with a single stimulus or pulse train. Each pulse train was composed of 20 impulses at each of the following frequencies; 10, 40 and 100 Hz. A 30-s interval occurred between each train. Stimulation pulses were delivered through an isolation unit, and the timing of the pulses was controlled by a microprocessor based digital timing device. The changes in CAP were recorded and transferred to a computer through the analog-digital converter and Acknowledge 3.7.1 data acquisition software (Biopac MP-150). The CAP parameters (the amplitude, the ½ falling time and the peak-time) were measured as shown in Figure 1B. The frequency-dependent inhibition (FDI) was determined by calculating the difference between CAP amplitudes following the first and last stimuli the pulse train, expressed as a percent of the control value. Kikusui 5529U digital storage oscilloscope, Grass S48 stimulator and stimulus isolation unit (SIU5), Grass P16 microelectrode AC/DC amplifier were used in the experiments.

Solutions: The Krebs' solution (in mM/L); 124.0 NaCl, 3.0 KCl, 1.3 NaH_2PO_4, 2.0 $MgCl_2$, 2.0 $CaCl_2$, 26.0 $NaHCO_3$, and 10.0 dextrose. Isotonic KCl (in mM/L); 120.0 KCl, 7.0 NaCl, 1.3 NaH_2PO_4, 2.0 $MgCl_2$, 26.0 $NaHCO_3$, and 10.0 dextrose. The isotonic sucrose; 320-mM sucrose. Deionised and bi-distilled water was used for the solutions. During the experiments all solutions were bubbled with a 95% O_2 and 5% CO_2 gas mixture and the pH of the solutions was adjusted to 7.4.

Photo assisted footprint recording and analysis

The video based experimental set-up has been described previously by Bervar, 2000. However, our experimental footprint system was constructed with a photo camera (Kodak DX7630) instead of video camera. The experimental set-up assembled under a glass table (50x100cm) with and intensity adjusted 100W electric lamp (Fig 1C). LCD screen on the photo camera used as a monitor. Rats were placed in the 10x20x20 cm sized Plexiglas box that was placed on the glass table one by one for the recording. The rats were kept in the box for 5 minutes for adaptation and after this period the photographs of the plantar

aspects were taken (1280x960 pixel). Neither filtering nor correction was made on the photos. The captured photos were analyzed with an Excell – Visual Basic based software developed in Department of Biophysics, University of Cukurova. Ratios of injured- uninjured hind feet parameters were determined calculating by averaging of the 5 photos of each rat (Fig 1D). We used 1-5 toe spread factor (TSF) instead of sciatic function index, and TSF was calculated giving the following formula below (OTS=operated toe spread, NTS=non operated toe spread).

TSF= (OTS-NTS)/NTS*100

Photos were captured from rats before the electrophysiological recordings in 2th, 4th, 6th and 8th weeks after the surgical operation. All the photo records were performed in a silent, dim place at room temperature.

Statistical analysis

All data are reported as mean ± standard error (S.E.M.). Mann-Whitney U tests were used to the differences. Differences were deemed statistically significant at $P< 0.05$.

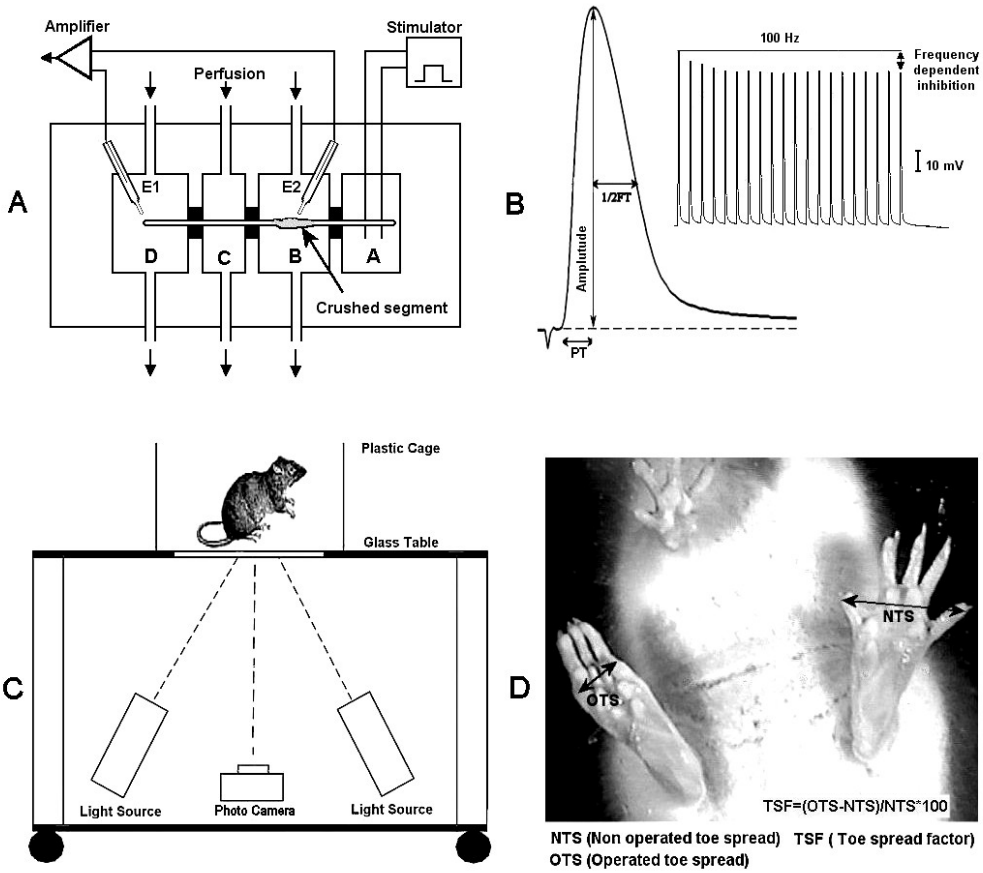

Fig.1. Schematic drawing of sucrose-gap apparatus (A). A typical compound action potential recorded by sucrose-gap technique with single stimulus. The amplitude of compound action potential, the ½ falling-time of CAP (1/2FT) and the peak-time of CAP (PT) are shown in the graphics which were obtained by supramaximal stimulation at single. Frequency-dependent inhibition (FDI) recorded by repetitive stimulation at 100 Hz frequency with 20 pulses described as shown in the figure (B). The photo assisted footprint system assembled a glass table, and intensity adjusted 100W electric lamp. LCD screen on the photo camera used as a monitor. (C). 1- 5 toe spread factor (TSF) was calculated using the formula as shown in the figure (E)

RESULTS

Photo assisted footprint analysis

In the 2nd, 4th, 6th and 8th days photo footprint recordings were performed before the electrophysiological recordings. In the 2nd weeks after the surgery, there was a complete loss of motor function (Fig. 2A). In the 2nd week after the crush, TSF were recorded as 45.4 ± 2.3% (Fig. 2A). TSF increased after 2nd week, and in the 4th week there was partial recovery in the motor functions. TSF were increased significantly to 64.8 ± 2.5%. Later, in the 6th and 8th weeks, TSF were increased to 86.1 ± 3.2% and 92.2 ± 3.5%, consequently.

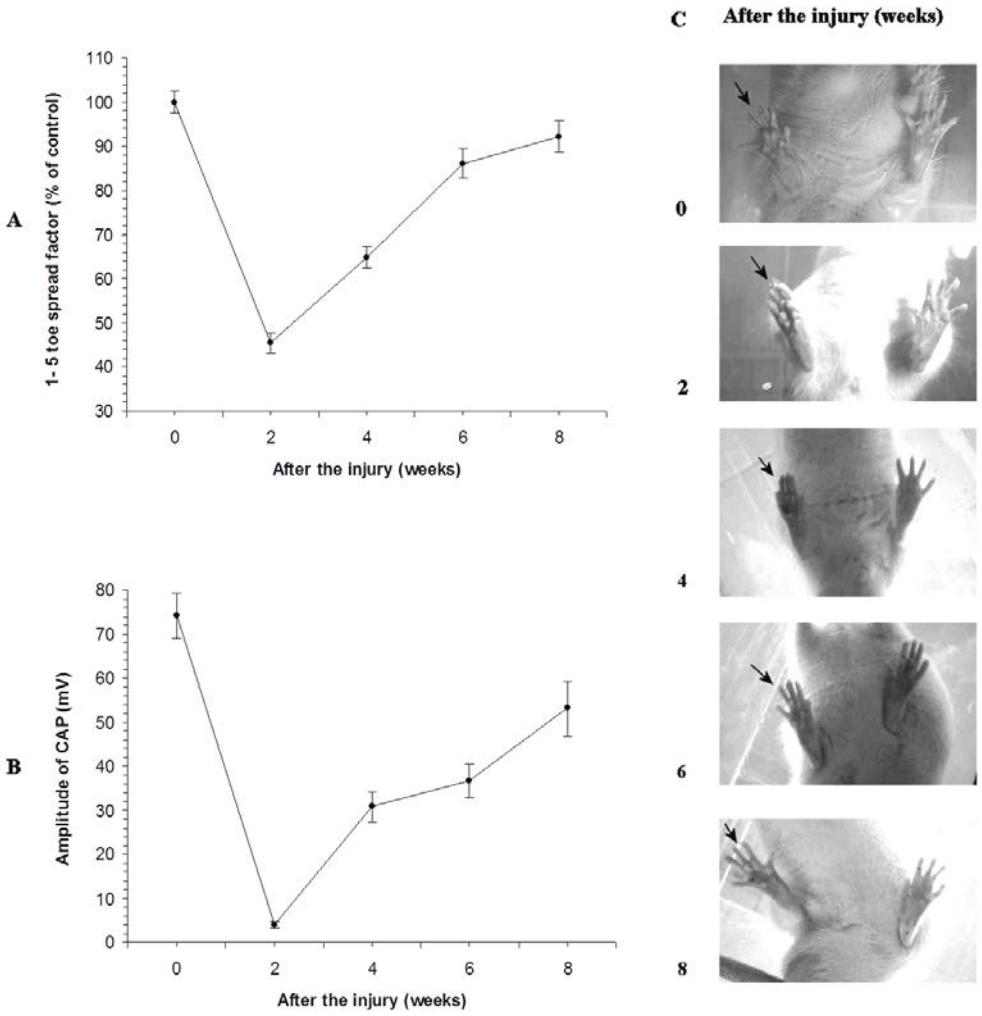

Fig.2. The graph shows function during the 8-week period of experiment, assessed by the TSI (A). The amplitudes of compound action potentials during the 8-week period of experiment, acquired by sucrose-gap technique (B).Ordinate: 1-5 toe spread factor, (% of control, 100 shows full recovery). Abscissa: Weeks. Data are presented as mean ± S.E.M. Photographs showing the plantar aspect of the rats after the sciatic crush injury during the 8-week period of experiment (C). Arrowheads show toe spreads of operated legs

Amplitudes of CAP

The amplitude of the CAP was recorded with sucrose gap technique and determined as 74.1 ± 5.2 mV in the intact group (Fig. 2B). 2 week later after the sciatic crush injury, the amplitude of CAP was too small (3.9 ± 0.7 mV). In the following weeks the amplitudes of CAPs were increased significantly, and reached to 53.1 ± 6.3 mV in 8th week.

Frequency-dependent inhibition

Sciatic nerve bundle was stimulated supramaximally with 100 Hz frequency with 20 pulse trains and frequency-dependent inhibition (FDI) was recorded (Fig. 3A). The FDI was determined as 94.1 ± 0.5 % in the intact group (Fig. 3A). In the 2nd week after the injury, FDI was 77,6 ± 2.2 %. In the following weeks FDI was increased to intact levels; 92.2 ± 1.5 % at the 8th week.

½ Falling-time of CAP

In the intact nerves, ½ FT of CAP was measured as 458.8 ± 21.7 ms. In the crushed nerves, 1/2FT was increased with a high ratio (Fig.3B). 1/2FT was extended significantly to 1571 ± 70 ms in the 2nd week after the injury. In the following weeks, 1/2FT was reached to 645 ± 90 ms at the 8th week.

Peak-time of CAP

In the intact nerves, the peak-time of CAP duration was determined as 407.5 ± 18.5 ms. The Peak-time of CAP was extended significantly to 1262 ± 60 ms in the 2nd week after the crush (Fig.3C). Later and recorded as 164.9 ± 8.0% (0.760 ± 0.040 ms) in the 35th day. In the following weeks the peak-time of CAP was reached to 665 ± 55 ms at the 8th week.

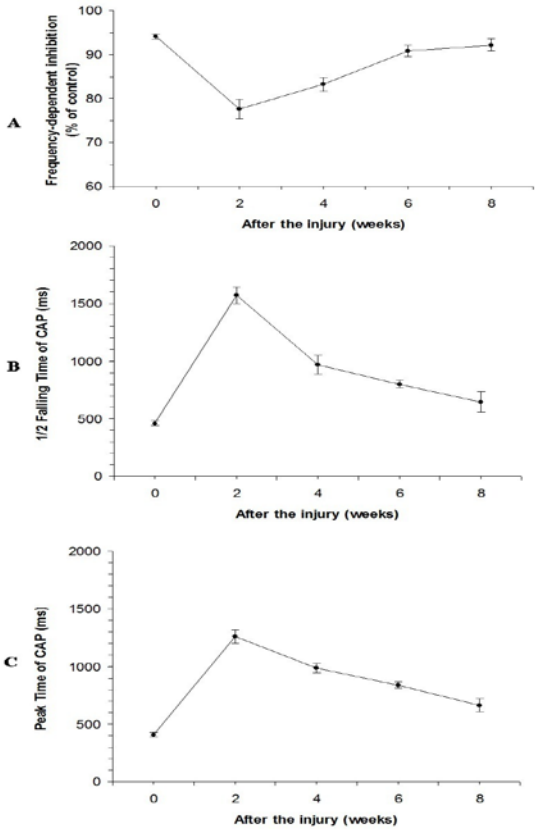

Fig.3. The graph show frequency-dependent inhibition. The data was obtained by repetitive stimulation at 100 Hz frequency with 20 pulses. The frequency-dependent inhibition was determined by calculating the difference between CAP amplitudes following the first and last stimuli the pulse train, expressed as a percent of the control value (A). Ordinate: % of control. The graphs show changes of the ½ falling-time and the peak-time of compound action potential after the sciatic crush injury during the 8-week period of experiment (B, C). Ordinate: The ½ falling-time of CAP or the peak-time of CAP (ms). Abscissa: Weeks. Data are presented as mean ± S.E.M.

DISCUSSION

Photo assisted footprint analysis, a method to determine combination of motor and sensory recovery of sciatic nerve regeneration. It is well known that the toes and the heel positions related

mainly two opposing forces, body weight and postural muscle tone. The recovery of muscle tonus after nerve injury is a constituent part of integral nerve and muscle functional recovery[9-11].

In our experiments, photo assisted footprint analysis was used for determination of functional recovery. Usually, footprint analysis results are expressed as sciatic function index (or static sciatic index as proposed by Bervar) by calculating with special formulas[10]. On the other hand, 1-5 toe spread (TSF) is a simple and the most useful parameter for measuring functional recovery after sciatic nerve injury. Therefore, our results were expressed as TSF[10,13].

In the study, complete loss of motor function was observed on operated leg of all animals after the sciatic nerve injury in the first 2 weeks. The injured rats were unable to spread all of the toes of the injured limb until the 3rd week. But a fast significant recovery occurred after the 2 week, and then in the 4th – 8th weeks, all the rats were able to spread all of their limb and toes. TSF represent the muscular tonus in the feet and so they indicate the ratio of the innervating nerves. For the reinnervation of the muscles, the crushed nerves must be regenerate from the crushed segment, and new axon sprouts must reach to the target muscles[9,10]. It is known that in the rats, axon regeneration speed after the crush is approximately 3-4 mm/day[1, 2]. In our experiments, sciatic nerves were crushed with a distance about 4-5 cm of the distal site. So, regenerating nerves reached to their target tissues within two weeks, and after the re innervating of target muscles TSF shows a rapid increase.

In our experiments, the sucrose-gap method was used to measure the electrophysiological activity in the crushed segment of the sciatic nerve bundle[14]. The CAP was measured with small amplitude in 2nd week. But the following 4th – 8th weeks, the amplitude of CAP was increased. The amplitudes of the CAP that is recorded by sucrose gap technique, reflects the sum of the action potentials that was formed in the nerve fibers in a bundle[14]. It is known that in the crushed nerves, axon membranes are damaged together with the ion channels, Schwann cells and other milieu[1, 2]. Later on, with the progression of regeneration, the number of regenerated axons increase and the ion channel composition becomes complete with the leading Na^+ channels that are mainly responsible for the formation of action potential[1]. So, improvement in regeneration increases the amplitude of CAP.

As it is known sciatic nerve is a complex bundle that is composed of myelinated-unmyelinated, fast-slow fibers. The findings show that frequency dependent inhibition was approximately 20% lower than intact nerves at 100 Hz conduction frequency in the 2nd week after the crush injury. Moreover, the ½ falling time of CAP and peak time of CAP were three fold longer than intact nerves in the 2nd week. In the following days, FDI and ½ falling time of CAP and peak time of CAP reached to intact nerve values. Longer ½ falling time and peak-time of CAP may indicate a decrease in the conduction velocity. As it is known, regenerating myelinated nerves have similar properties with the unmyelinated ones at the beginning of regeneration. Also, the conduction velocity decreases approximately 5 fold and the duration of absolute refractory period increases 2 to 5 fold[5,6,21]. In the high frequency repetitive stimulation, action potential does not develop if a stimulus is given in the refractory period of the preceding one. However, in the following days of regeneration, developing of myelination, thickening of the nerve fibers, accumulation of Na^+ channels in the Ranvier nodes, may cause increase conduction velocity and changing CAP parameters to intact levels.

In the present study, functional and electrophysiological development in the crushed rat sciatic nerves were evaluated using photo assisted footprint analysis and sucrose-gap methods together. We observed complete motor function loss in two weeks after the crush injury, but the amplitude of CAP increases significantly until the two weeks. However, TSF rapidly increased and displayed almost full functional

recovery in the 4th week, but frequency dependent inhibition, peak-time of CAP and ½ falling time of CAP did not reach to intact values in the 4th or 8th weeks.

We conclude that photo assisted footprint analysis method and sucrose-gap technique, which are useful functional and electrophysiological methods to produce complementary results each other in the investigation of experimental peripheral nerve regeneration.

Acknowledgments

This study was supported partially by grants from the TUBITAK-SBG (106S220).

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geliş tarihi/received :12.06.2012
kabul tarihi/accepted:29.06.2012