

Effect of FK506 administration in alpha motor neurons after primary and delayed repair of the sciatic nerve

Umut Özsoy¹, Fatoş Belgin Yıldırım¹, Bahadır Murat Demirel², Arzu Hızay¹, Levent Sarıncıoğlu¹, Necdet Demir³, Gamze Tanrıöver³, Bikem Süzen¹, Nurettin Oğuz¹

¹Department of Anatomy, Faculty of Medicine, Akdeniz University, Antalya, Turkey

²Department of Anatomy, Faculty of Medicine, SANKO University, Gaziantep, Turkey

³Department of Histology and Embryology, Faculty of Medicine, Akdeniz University, Antalya, Turkey

Abstract

Objectives: FK506 is an effective immunosuppressive drug for treating graft rejection in transplant patients. However, the neuroregenerative effect of FK506 has been well described in the literature. The aim of this study was to investigate the effects of FK506 in alpha motor neurons after primary and delayed repair of sciatic nerve.

Methods: Rats (n=72) were divided into 6 groups. Control, sham-operated, primary repair FK506 (-), primary repair FK506 (+), delayed repair FK506 (-), and delayed repair FK506 (+) groups.

Results: After injury, the normal structure of the motor neuron perikarya was maintained by primary repair in the FK506 (+) group. In the delayed repair group, beneficial effect of FK506 was found to be less effective. The SFI value reached -50 recovery level in the FK506-treated group earlier than those of not FK506-treated groups.

Conclusion: Beneficial effect of FK506 has been approved by functional and ultrastructural data in both of primary and delayed repair groups.

Keywords: delayed repair; FK506; primary repair; regeneration; sciatic nerve

Anatomy 2016;10(1):30–39 ©2016 Turkish Society of Anatomy and Clinical Anatomy (TSACA)

Introduction

Axotomy of the peripheral nerves causes retrograde cell death in the dorsal root ganglion neurons. Following peripheral nerve injury, the axotomized motor and sensory neurons undergo a series of retrograde degenerative changes which may result even in neuronal death.^[1-4] The degenerative changes are caused by injury-induced interruption of the flow of neurotrophic factors from periphery to neuronal body by retrograde transport.^[5-9] Degenerative changes occurring following peripheral nerve injuries vary with the subject's age, neuron type, lesion level, postoperative survival time, and type of injury. For example, in motor and sensory neurons of newborn animals, cell death has been reported in almost all types of peripheral nerve

injury.^[4,10] However, after peripheral nerve transection, degeneration has been reported^[11-14] in dorsal root ganglia^[15] as well as motor neurons of cranial nerves and spinal cord.^[16-19] Some authors^[19-23] reported no loss of motor neurons in peripheral nerve crush injury, some studies also reported motor neuron survival following permanent peripheral nerve cut or limb amputation.^[22,24,25]

FK506 (also known as Tacrolimus, Prograf) is a powerful immunosuppressive drug for treating graft rejection and is reputed to have a superior potency relative to Cyclosporine A (CsA) in transplant patients for prevention of graft rejection.^[26] FK506 is known to inhibit calcineurin-mediated T-cell activation by forming a complex with FK506 binding protein (FKBP-12).^[27,28] However, FK506 also has a well-studied neuroregenerative effect

that may be related to FKBP-12 operating through a separate mechanism.^[29]

Effect of FK506 on different models has been well documented in the literature. FK506 has been shown to exert neuroprotective effects in degenerative diseases,^[30] ischemic insults,^[31–33] spinal cord injury,^[34,35] and neurite outgrowth in vitro.^[36,37] Furthermore, treatment with FK506 has also been reported to enhance the rate of axonal regeneration in crush injury model^[38–41] and in a peripheral nerve grafted into the injured spinal cord.^[42] FK506 has been shown in many studies to enhance recovery after immediate^[43] or delayed^[29,38,44] nerve repair. FK506 promotes neurite outgrowth in vitro and its efficiency in enhancing nerve regeneration and accelerating functional recovery has been demonstrated in transection,^[45–48] crush,^[39,40,43,49] isograft,^[50–52] and allograft models.^[53–55]

In clinical practice, diagnosis and treatment of patients with peripheral nerve injuries is often delayed. The effect of a delayed nerve repair on nerve regeneration and muscle reinnervation is still not well understood. This is surprising, because in the clinical routine an immediate reconstruction of the lesioned nerve is seldom possible. In contrast to typical animal models for nerve regeneration studies, there is a certain denervation time of the affected nerve between lesion and reconstruction. The results of the few animal studies using different nerves, animal models, and methods are contradictory.^[56–61] Some of them showed that an immediate nerve suture was superior to any delayed nerve suture, whereas others reported no differences. Therefore, studying on a more relevant model of nerve reconstruction is important.

Although immediate subcutaneous applications of FK506 after incision have been reported as the most effective method,^[29] delayed or intermittent applications have also been reported as effective methods.^[62] In a recent report, it has been demonstrated that the delayed administration of FK506 after surgery (even a 7-day-delay) causes reduction in axonal regeneration.^[29] In literature, beneficial effects of the FK506 have been reported in peripheral

nervous system. However, effect of FK506 on alpha motor neurons has not been reported. Therefore, in the present study we aimed to study effects of FK506 on alpha motor neurons after primary and delayed repair of sciatic nerve.

Materials and Methods

Animals

A total of 72 female Wistar rats (200 to 250 g) were used in the present study (Table 1). We used female rats, since it has been reported that testosterone accelerates the regeneration of peripheral nerves.^[63,64] The rats were divided into 6 groups as follows: Group 1, control; Group 2, sham-operated; Group 3, primary repair FK506 (-); Group 4, primary repair FK506 (+); Group 5, delayed repair FK506 (-), and Group 6, delayed repair FK506 (+). Six animals were housed per cage and maintained on a 12:12 h light-dark cycle; lights on from 7.00 to 19.00. Food and water were provided *ad libitum*. All procedures were reviewed and approved by the Animal Care and Usage Committee of Akdeniz University (protocol number 04-17).

Two subjects from the FK506 (+) and FK506 (-) groups each were excluded from the study due to postoperative death within a week. New subjects were operated in place of these, and the necessary postoperative care and FK506 applications were performed.

Sciatic nerve injury

Before surgery, animals were anesthetized with intramuscular injection of a mixture of Xylazine HCl (15 mg/kg) and Ketamine (100 mg/kg). The left side of the hindlimb was shaved and swabbed with antiseptic solution. A longitudinal cutaneous incision was made in the back of the thigh. Dissection was carried out along a plane separating the hamstring and gluteal muscles to expose the sciatic nerve. Careful dissection was performed to isolate the sciatic nerve from the surrounding connective tissue over a length of 2 to 2.5 cm. The sciatic nerve was unilaterally injured by microscissors (Figure 1a–c).

Table 1
Classification and number of study groups.

	n	Cut injury	Primary repair	Delayed repair	FK506 treatment
Group 1: control	12				
Group 2: sham-operated	12				
Group 3: primary repair	12	+	+		-
Group 4: primary repair + FK506	12	+	+		+
Group 5: delayed repair	12	+		+	-
Group 6: delayed repair + FK506	12	+		+	+

In the primary repair group, the cut nerve stumps were coapted (totally 4 sutures) with 10.0 suture material (**Figure 1d**). The wound was closed with 2-0 suture material and rats were allowed to recover. In the sham-operated group, after a longitudinal cutaneous incision, the sciatic nerve was exposed and the wound was closed without sciatic nerve injury. In the delayed repair group, the skin was closed following the nerve cut. At the end of a seven-day delay period, the sprouted axons in the proximal nerve stump were pruned to refresh the cut. After this procedure, the cut nerve stumps were coaptated the same way as in the primary repair group.

FK506 administration

5 mg/kg/day subcutaneous FK506 (Prograf, Eczacıbaşı, Istanbul, Turkey) administration was performed from the day of sciatic nerve injury until the day of sacrifice. The FK506 administration was not interrupted during

postoperative days (even at the weekends). The same volume of saline was administered to the vehicle-treated animals (Groups 3 and 5). Animals were weighted weekly to adjust dose of FK506 for each animal.

Evaluation tests

Walking pattern analysis

Motor function recovery was monitored by analyses of free walking patterns. This method was originally described by De Medinaceli et al.,^[65] and later modified by Carlton and Goldberg^[66] and Bain et al.^[67] The overall index of sciatic nerve function was used as a parameter to evaluate the recovery of coordinated motor function of the injured hind paw. The test procedure is as follows: both hindpaws of the rats were touched on a stamp containing ink. Then, rats were allowed to walk on a corridor (80 cm long, 7 cm wide, and inclining 10 degrees) covered by pho-

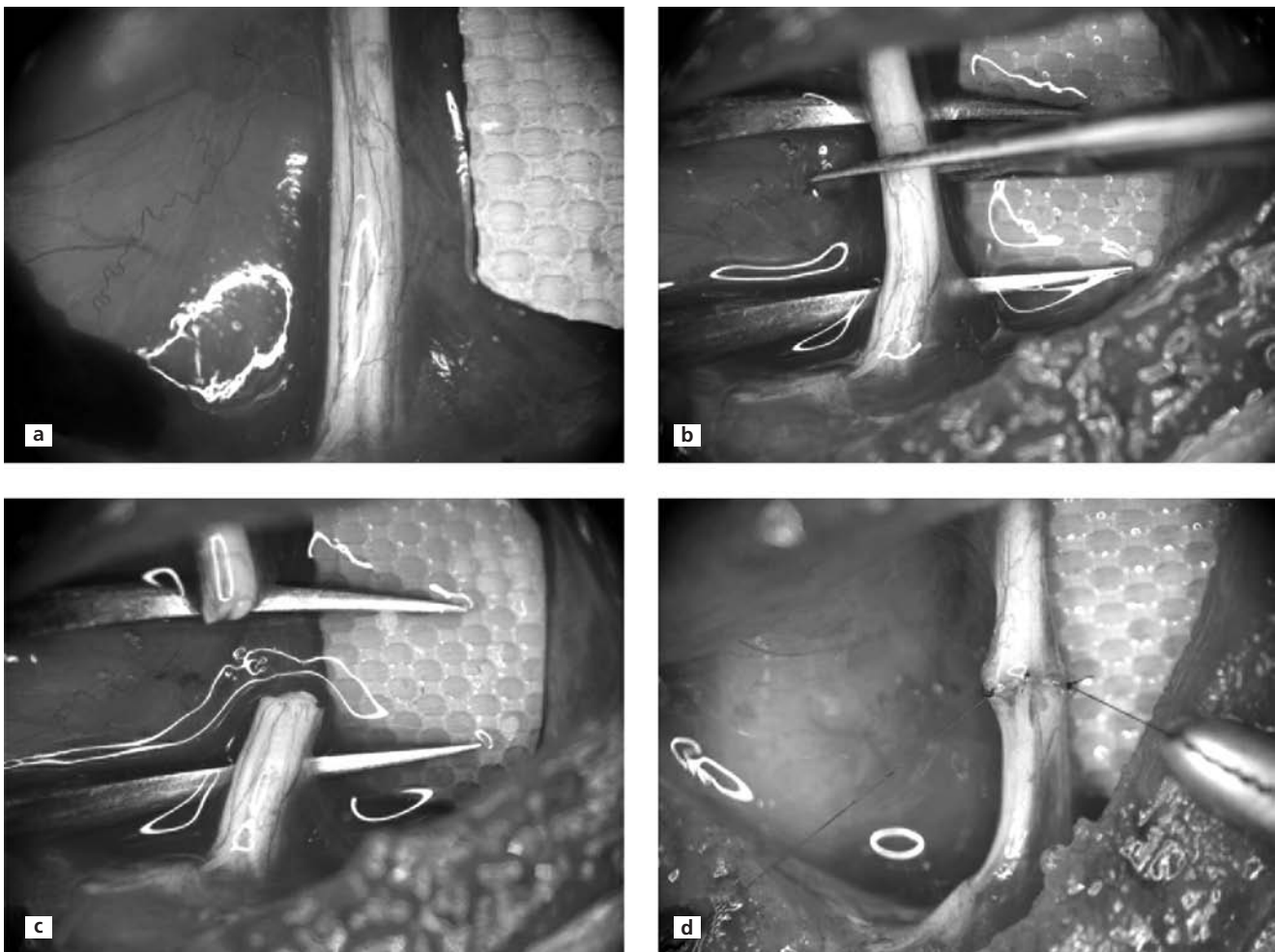


Figure 1. Nerve injury. (a) Normal appearance of the sciatic nerve, (b, c): nerve cut, (d) epineurial nerve coaptation. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

tocopy paper. From the footprints, the sciatic function index (SFI) was calculated using the formula developed by Bain et al.^[67] An SFI of 0 was considered normal, whereas an SFI of -100 meant total impairment. An experienced observer analyzed the walking patterns on the 1st, 2nd, 3rd, 4th, 5th, and 6th postoperative weeks.

Sensory function

Functional sensory recovery was analyzed by pinch test. The sole of the foot was pinched using a forceps. A gradual scale with four levels was used to assess withdrawal reflex (sensory recovery as: no response (Grade 0), mild response (Grade 1), moderate response (Grade 2), and full response (Grade 3). Animals showing full withdrawal response to pinch (Grade 3) were noted for further statistical analysis. Withdrawal response of animals was noted on the 1st, 2nd, 3rd, 4th, 5th, and 6th postoperative weeks.

Tissue collection

On the last postoperative day, rats were anesthetized with a combination of Ketamine (Ketalar®) (100 mg/kg, IM) and Xylazine HCl (Rompun®) (15 mg/kg, IM) diluted in saline. Rats were perfused transcardially with 250 ml heparinized saline and spinal cord was dissected free. Spinal cord samples (approximately 1 cm) were harvested from the level of lumbar intumescence. Care was taken not to damage spinal cord tissue while dissecting the surrounding bony structures. Sciatic motor neuron pool was sampled after transverse sectioning of the lumbar intumescence. Sciatic nerve samples were harvested 0.5 cm below the lesion site and immediately transferred to routine electron microscopic tissue preparation procedure.

Electron microscopic analysis

Spinal cord samples (each 1 mm³) belonging to lumbar intumescence were fixed with 2% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.2) at +4° for two hours. The tissue samples were post-fixed in 1% osmium tetroxide (OsO₄) solution for one hour. Samples were dehydrated through a gradually increasing series of ethanol and embedded in Araldite resin (Araldite CY 212, 91 ml + dodecenyl succinic anhydride (DDSA) 84 ml+N,N-dimethylbenzylamine (BDMA) 3.5 ml, TAAB Company, Aldermaston, UK). Semi-thin (1 micrometer) and ultra-thin (40–60 nm) sections were obtained using diamond knives on a Leica ultramicrotome (Leica Ultracut, UCT, Leica MZ6; Leica Microsystems GmbH, Wetzlar, Germany). Sections were examined to determine the ventral horn for motor neuron pool. Then, ultra-thin sections were obtained from area of interest. Ultra-thin sections were collected on copper grids (Mesh 100, TAAB, England) coated with Formvar 15/95 Resin (EMS-Electron Microscopy Sciences, Fort Washington, PA,

USA). In semi-thin sections (1 micrometer), routine toluidine blue staining method was applied for orientation. In ultra-thin sections, double-contrast staining was applied with uranyl acetate (100 ml methanol and 5 g uranyl acetate) and Reynold's lead nitrate solution. Ultra-thin sections were examined using a transmission electron microscope (Leo 906E; Zeiss, Jena, Germany).

Data analysis

All data (mean ± standard deviation) were analyzed with SPSS Statistical Package for Social Sciences (SPSS) software (version 11.0; SPSS Inc, Chicago, IL, USA). A p value less than 0.05 was considered as statistically significant. Comparison of SFI data was done using one way ANOVA (with Tukey's post hoc test) and Pinch test data was done by chi-square test.

Results

SFI values

The print length (PL) of the animals in all experimental groups was considerably longer, and toe spreading and intermediate toe spreading was narrower on the injured site on the day following injury. The SFI values were -90.65 ± 5.29 , -99.65 ± 6.42 for Group 3 (primary repair) and Group 5 (delayed repair), respectively. The SFI value in Group 3 (primary repair) and Group 5 (delayed repair) could not recover to the -50 values even at the end of the 6th postoperative weeks (Figure 2). FK506 treatment

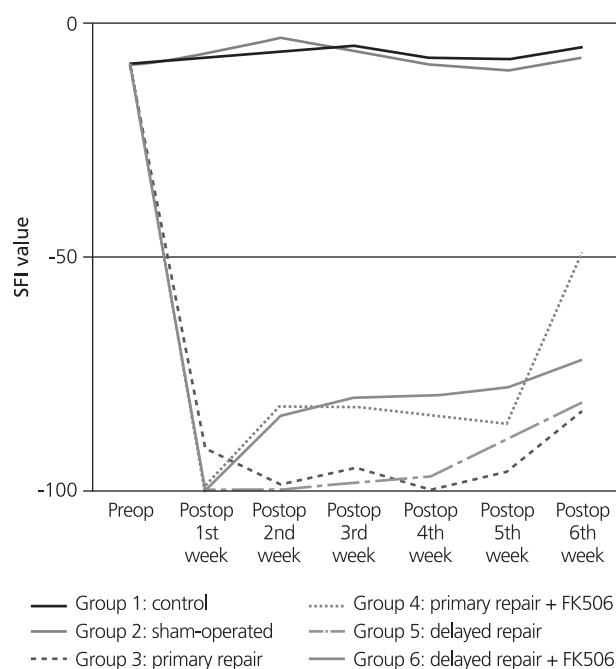


Figure 2. Sciatic function index values of groups.

affected the onset week of the -50 level of SFI among the FK506 treated groups, i.e. while Group 4 (primary repair + FK506) reached -50 levels Group 6 (delayed repair + FK506) could not reach at the 6th postoperative week. At the end of the 6th postoperative week no significant difference was observed between Group 2 (sham-operated) and Group 4 (primary repair + FK506).

Onset day of withdrawal response to pinch

The onset day of withdrawal to pinch appeared at the 3rd postoperative week in Group 4 (primary repair + FK506) and Group 5 (delayed repair). In this week, only two animals displayed Grade 3 withdrawal to pinch. However, seven, two, and three animals showed withdrawal to pinch in Group 4 (primary repair + FK506), Group 5 (delayed repair), and Group 6 (delayed repair + FK506), respectively (Figure 3). At the end of the 6th postoperative week no significant difference was observed between Group 2 (sham-operated) and Group 4 (primary repair + FK506).

Electron microscopic evaluations

The ultrastructure of sciatic nerve samples of the control and sham-operated groups displayed normal structural organization (Figure 4). Ventral horn samples in the control group were compared to those of primary repair and delayed repair groups with or without FK506 treatment. The ultrastructural morphology of the ventral horn in the primary repair group with FK506 seemed to be similar to the control groups. There was no apparent structural damage on the pericaryon of motor neurons. Also, no defect or edema was observed in the neuropil area and neurons. However, clear oedematous gaps wrapping the pericaryons were evident in Group 3 (primary repair). In this group, capillary diameters were observed to be narrowed. Moreover, oedematous areas were observed in the astrocytic feet.

The preservative effect of FK506 was revealed in the delayed repair group as well as the primary repair group ultrastructurally. Also, no neuropil oedemas or deformation areas were evident in Groups 4 and 6. On the other

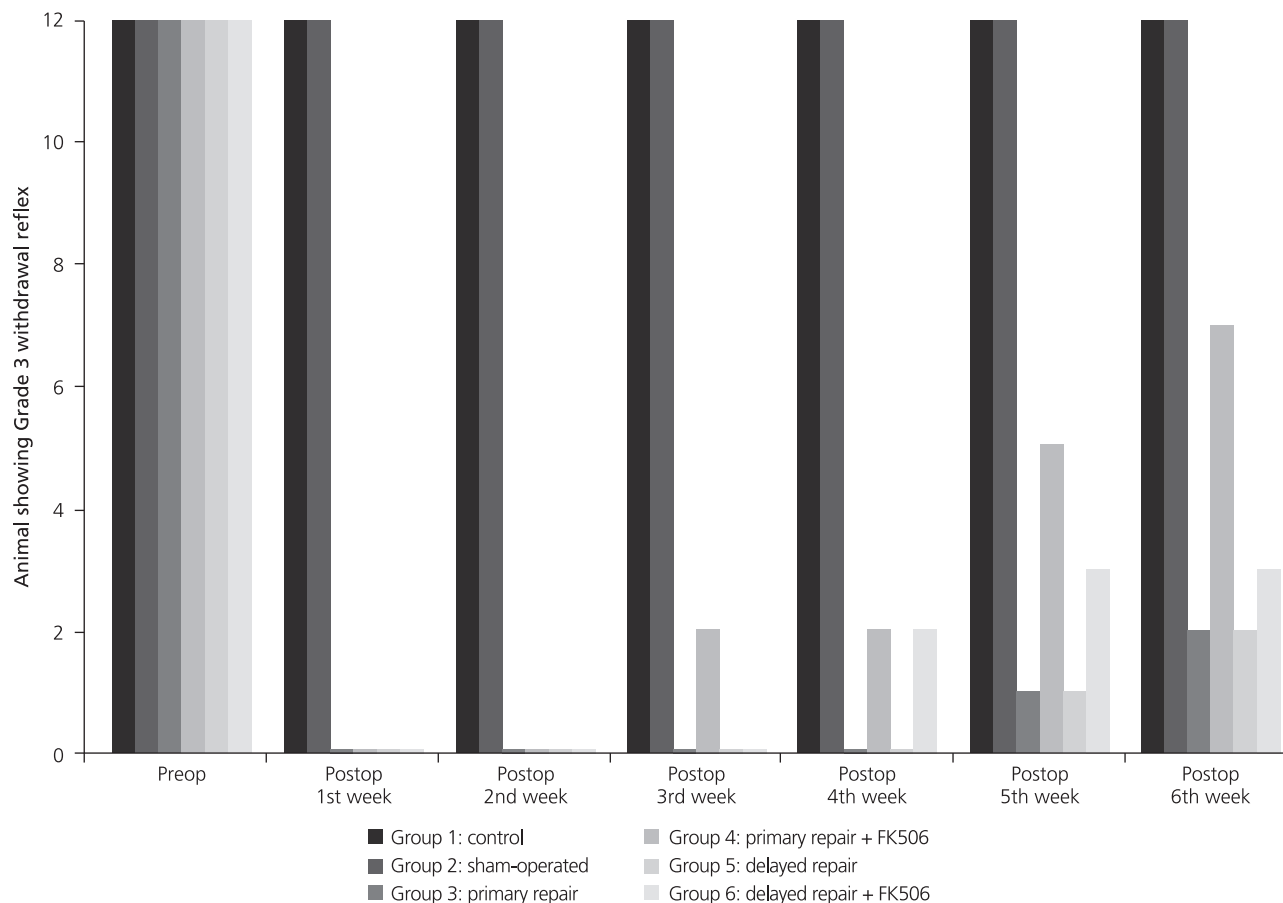


Figure 3. Number of animals showing Grade 3 withdrawal response.

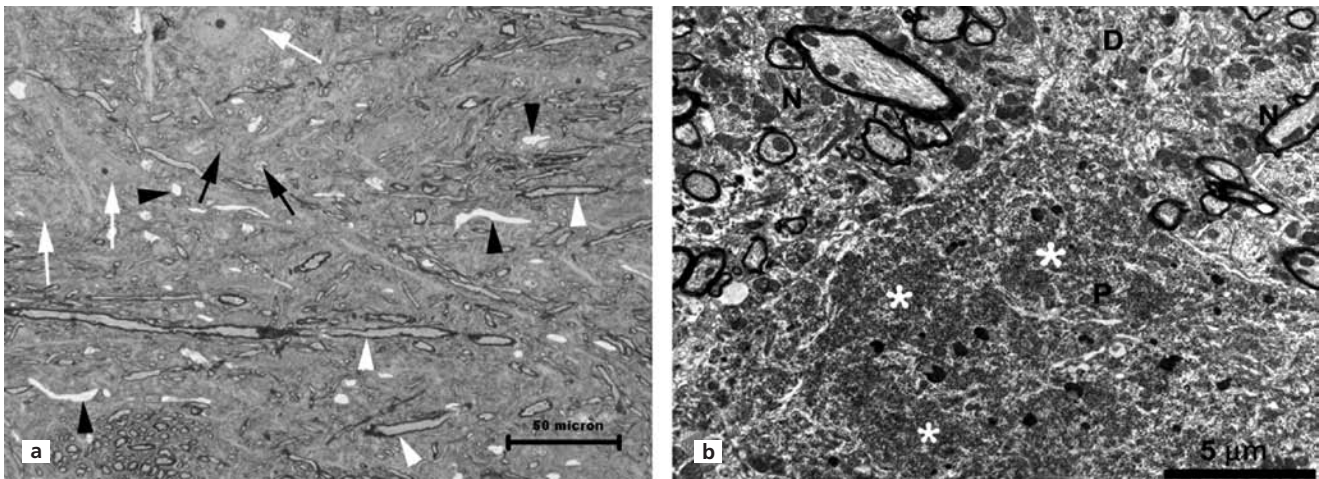


Figure 4. The light and transmission electron micrographs of motor neurons in the control group. (a) Motor neurons (white arrows), glia (black arrows), capillaries (black arrowheads), myelinated axons (white arrowheads) and different sizes of the capillaries are shown in semithin sections. (b) The ultrastructural feature of neuropils (N) were observed with neurons with Nissl bodies (*) in cytoplasm and dendrites. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

hand, FK506 was not properly effective on the ultrastructure of neurons (Figure 5).

Discussion

A correlation between time of nerve repair and functional recovery was reported in a number of studies.^[68-71] Time-dependent changes that affect the clinical outcome have also been reported following nerve injury.^[47,48,58,69-73] Therefore, relationship between the time of injury, surgical repair and initiation of drug therapy are important factors in usage of agents with neurodegenerative and/or neuroprotective effects. In the present study, we limited postoperative period as 6 weeks to observe the effects of FK506 on functional, ultrastructural properties of nerve recovery. This postoperative time period was also used in other studies.^[43,74-77]

Sulaiman et al.^[48] investigated the effect of FK506 treatment in chronic denervation and chronic axotomy models which were formed by a total transection of the tibial and common peroneal nerves. In the chronic axotomy model, at the end of a 2-month delay, the proximal stump of the tibial nerve was coaptated to the freshly cut distal stump of the peroneal nerve. In another model of chronic denervation, after a 2-month of delay, the proximal stump of the peroneal nerve was coaptated to the freshly cut distal stump of the tibial nerve. Afterwards, they reported an increase in the number of HRP-labeled motor neurons, number of axons and myelin sheath thickness after daily administration of 5 mg/kg FK506 in chronic axotomy model. Although the study of Sulaiman et al.^[48] was not applicable in clinical practice, their

results are important in terms of giving an idea about the effects of FK506.

In clinical practice, diagnosis and treatment of patients with peripheral nerve injuries is often delayed. Coaptation of the transected nerve stumps cannot be performed immediately after incision. However, in the experimental setups, neuroregenerative/neuroprotective agents are often applied soon after nerve cut injury and coaptation. In the present study, we applied delayed nerve repair model in line with the frequently encountered clinical scenario, and investigated the ultrastructural effects of FK506 on the alpha motor neurons. We found that FK506 treatment resulted in normal motor neuron structure. This finding was not observed in FK506 (-) group. Additionally, in the delayed repair group, in which nerve repair was applied 7 days after the injury, beneficial effect of FK506 was lower than those of the primary repair group. In a recent study, Brenner et al.^[29] investigated the neuroregenerative effect of FK506. In their study, they applied FK506 after primary and delayed repair and found that FK506 administration after delayed repair did not cause an increase in the nerve regeneration. Additionally, they showed a decrease in the number of retrogradely labeled motor neurons.

Ma et al.^[78] found 21–31% loss of motor neurons at 8 and 16 weeks following C7 spinal nerve injury. Effects of the delayed repair after nerve cut injury were not only investigated on the spinal nerves, but also on the cranial nerves. Guntinas-Lichius et al.^[79] examined the effects of delayed repair using the hypoglossal-facial anastomosis model to reanimate the facial nerve. In addition, they reported that this model was more realistic for repair of

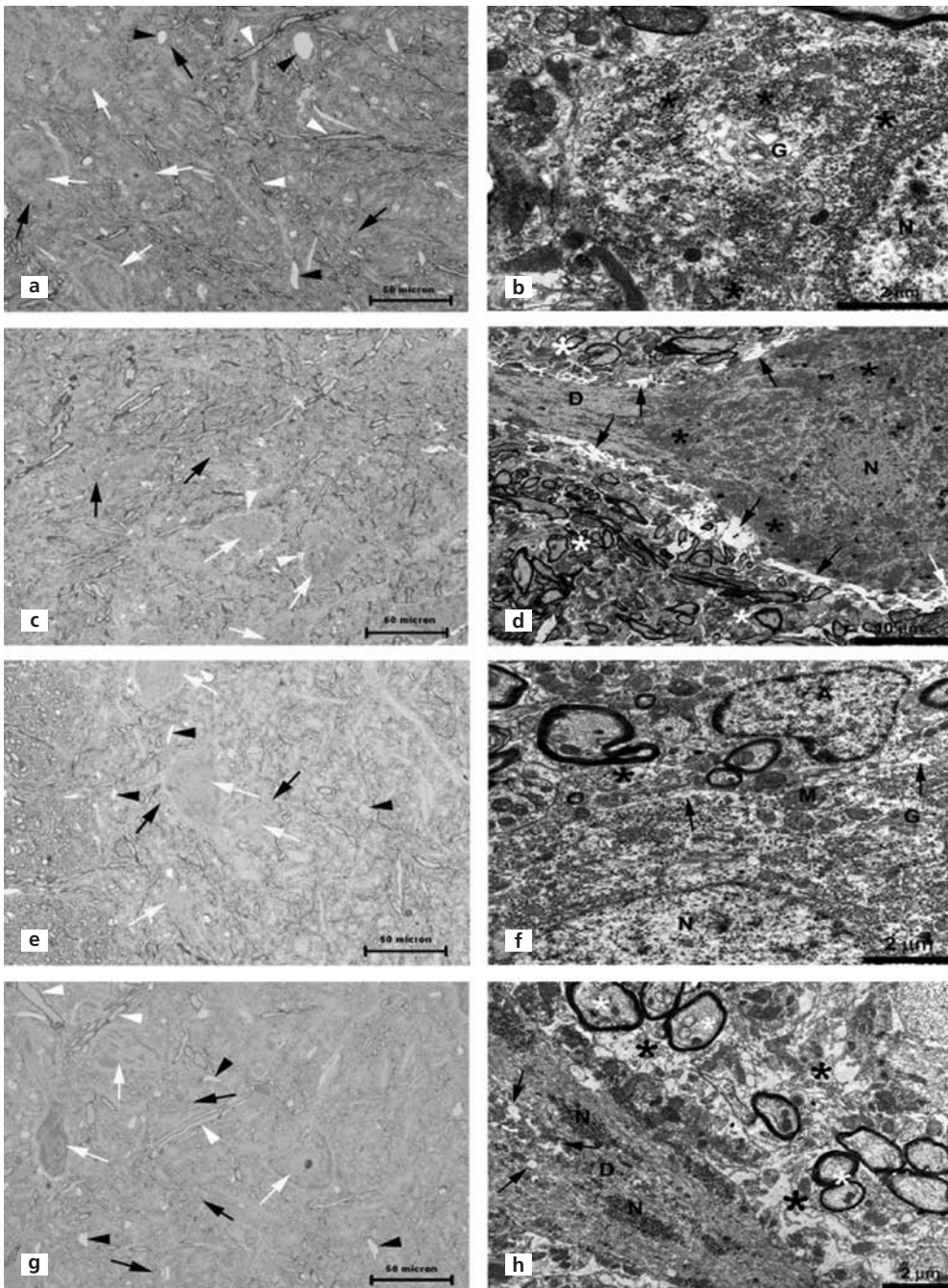


Figure 5. Representative pictures of motor neurons in the experimental groups obtained by light and electron microscopy. (a) Motor neurons (white arrows), glia (black arrows), myelinated axons (white arrowheads) and capillaries can be seen in semithin sections in primary repair with FK506 groups. (b) Motor neurons with Nissl bodies (*), enlarged Golgi cisterns (G) and euchromatic nuclei (N) in ultrathin sections in the same area. (c) The semithin sections showing motor neurons (white arrows) consisting of enlarged edematous areas (white arrowheads) and glia nuclei in neuropils of the primary repair without FK506 group. (d) Transmission electron micrographs of surrounded motor neurons show dense edematous areas and also separation areas (black arrows) in the same group. In addition, cytoplasmic Nissl bodies (*) and euchromatic nuclei (N) with healthy main structural components are evident. (e) Motor neurons (white arrows), glia (black arrows), and also capillaries (black arrowheads) can be seen in normal structural organization in semithin sections of delayed repair with FK506 group. (f) The area around the perikaryon reveals separations in some places (arrows). Neuropils (*), astrocytes (A), mitochondria (M), Golgi apparatus (G) and euchromatic nuclei have normal appearance in the delayed repair with FK506 group. (g) Motor neurons (white arrows), glia (black arrows), myelinated axons (white arrowheads) and capillaries (black arrowheads) display structural organization in semithin sections of delayed repair with FK506 group. (h) The neuropils (*) have dense edema and deformation as displayed in trans-

mission electron micrographs of the delayed repair without FK506 group. Abnormally enlarged cisterns of granulated endoplasmic reticulum (arrows) and also Nissl bodies (N) in dendrites (D) were spectacular observations of ultrastructure in these groups. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

a predegenerated nerve, as a certain time was allowed after cut of the facial nerve. Meanwhile, Wallerian degeneration occurred on the distal part of the nerve, debris of myelin was phagocytized, and atrophic changes were evident in the target muscle. They reported that the coaptation of the freshly cut hypoglossal nerve with predegenerated facial nerve would be a more appropriate

way to investigate the effect of the delayed repair. Using this model, the researchers coaptated the freshly cut hypoglossal nerve to the predegenerated facial nerve after the postoperative days 7, 14, 56, 112, and 224. The delayed repair was reflected by an increase in neurons labeled with HRP, whereas decreases in cross-section area of muscle of delayed repair groups.

Sobol et al.^[44] studied the effects of FK506 administration after primary nerve repair and reported that nerve regeneration was significantly increased after administration within 3 days, while a 5 day delay in the nerve repair caused little or no effect. Therefore, it was concluded that FK506 was effective when given immediately after injury. In our study, delayed administration of FK506 after repair displayed a beneficial effect on the motor neuron ultrastructure, but primary repair was more effective when FK506 treatment started immediately after injury.

Conclusion

FK506 is used to provide functional recovery at early period of peripheral nerve injury. Beneficial effects of FK506 were determined in both groups of primary and delayed repair. Application of the FK506 treatment after primary repair gave better ultrastructural and functional results. Our literature search revealed no article, describing beneficial effects of FK506 administration on sciatic motor neurons after primary and delayed repair.

Acknowledgements

This study was supported by Akdeniz University Research Fund (2007.01.0103.004).

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Online available at:
www.anatomy.org.tr
doi:10.2399/ana.15.038
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Correspondence to: Fatoş Belgin Yıldırım, PhD
Department of Anatomy, Faculty of Medicine,
Akdeniz University, 07070, Antalya, Turkey
Phone: +90 242 227 44 85
e-mail: yildirimfb@akdeniz.edu.tr

Conflict of interest statement: No conflicts declared.

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