The repair of peripheral nerve injuries is still one of the most challenging tasks and concerns in neurosurgery. Nerve autograft remains a gold standard; however, there are several drawbacks such as sacrifice of functioning nerves, loss of sensation, and mismatch between nerve and graft. Vein grafting has shown promising results in directing axons from the proximal to the distal stump and creating a local microenvironment. The development of therapeutic agents that can promote the rate of nerve regeneration and enhance the degree of functional recovery after injury is important. By accelerating axonal regeneration, such agents could diminish the consequences of denervation on target organs (muscle atrophy, loss of sensory receptors, and denervation hypersensitivity) and result in more efficient functional recovery (following reinnervation). FK506 is a FDA-approved immunosuppressant used primarily for the prevention of allograft rejection after transplantation. Recently, studies have been focusing on its role as a neuroprotectant and neurotrophic agent that promotes functional recovery and reinnervation following peripheral nerve injury. Systemic administration of FK506 in combination with graft therapy or tube repairing has been shown to enhance the rate of nerve regeneration and promote the degree of functional recovery. Nephrotoxicity, hypertension, hyperesthesia, muscular weakness, and gastrointestinal symptoms are side effects of systemic administration of FK506 because of its nonselective mechanism of action. Both the potential side effects and the immunosuppressive properties may "preclude the broad clinical use of FK506 to speed nerve regeneration." Moreover, in repair using a silicone tube, the effect of systemic administration was found to be only margin-

Effects of topically administered FK506 on sciatic nerve regeneration and reinnervation after vein graft repair of short nerve gaps


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Object. Despite the development of various nerve coaptation materials and techniques, achievement of desired functional peripheral nerve regeneration is still inadequate, and repair of peripheral nerve injuries is still one of the most challenging tasks and concerns in neurosurgery. The effect of an FK506-loaded vein graft as an in situ delivery system for FK506 in bridging the defects was studied using a rat sciatic nerve regeneration model.

Methods. A 10-mm sciatic nerve defect was bridged using an inside-out vein graft (IOVG) filled with 10 μl of a carrier-drug dilution (10 ng/ml FK506) in the IOVG/FK506 group. In the IOVG control group, the vein was filled with the same volume of carrier dilution alone. The regenerated fibers were studied 4, 8, and 12 weeks after surgery.

Results. Functional study confirmed faster recovery of the regenerated axons in the IOVG/FK506 group than in the IOVG group (p < 0.05). There was a statistically significant difference between the mean gastrocnemius muscle weight ratios of the IOVG/FK506 and IOVG control groups (p < 0.05). Morphometric indices of regenerated fibers showed that the number and diameter of the myelinated fibers were significantly higher in the IOVG/FK506 group than in the IOVG control group. Immunohistochemical analysis showed more positive immunoreactivity to S100 protein in the IOVG/FK506 group than in the IOVG control group.

Conclusions. When loaded in a vein graft, FK506 resulted in improvement of functional recovery and quantitative morphometric indices of sciatic nerve. Topical application of this readily available agent offers the benefit of cost savings as well as avoiding the complications associated with systemic administration.

(Objective): Despite the development of various nerve coaptation materials and techniques, achievement of desired functional peripheral nerve regeneration is still inadequate, and repair of peripheral nerve injuries is still one of the most challenging tasks and concerns in neurosurgery. The effect of an FK506-loaded vein graft as an in situ delivery system for FK506 in bridging the defects was studied using a rat sciatic nerve regeneration model.

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Key Words • peripheral nerve repair • sciatic • FK506 • topical application • rat

Abbreviations used in this paper: IOVG = inside-out vein graft; PBS = phosphate-buffered saline; SFI = Sciatic Function Index.
al.21 Thus, a localized, effective, and sustained delivery is crucial for a potentially broader use of FK506. The topical effect of FK506 on peripheral nerve repair had not been well investigated to date. Because of promising beneficial effects of vein grafting as an easily available conduit and the neuroregenerative and immunosuppressive effects of FK506, the objective of the present study was to investigate the use of a FK506-loaded vein graft as an in situ delivery system of FK506 in bridging the defects in a rat sciatic nerve transection model. Assessment of nerve regeneration was based on functional (walking track analysis), histomorphometrical, and immunohistochemical (Schwann cell detection by S100 expression) assessment at 4, 8, and 12 weeks after surgery.

Methods

Experimental Design

Fifty-four male white Wistar rats weighing approximately 270 g were divided into 3 experimental groups (n = 18), randomly: a sham-surgery group (Sham), an IOVG control group (IOVG), and an FK506-treated group (IOVG/FK506). Each group was further subdivided into 3 subgroups of 6 animals each. Thirty-six male white Wistar rats weighing 300–350 g were used as vein graft donors. Two weeks before and during the entire experiments, the animals were housed in individual plastic cages with an ambient temperature of 23°C ± 3°C, stable air humidity, and a natural day/night cycle. The rats had free access to standard rodent laboratory food and tap water.

Preparation of FK506

The dose of FK506 was determined according to the method described by others.6 Briefly, the original solution of FK506 (Prograf, Astellas Pharma) was 5 mg/ml, so 2 dilutions were performed to reduce the concentration to 10 ng/ml: carrier dilution (20 μl original FK506 into 10 ml sterile olive oil to give 10 μg/ml) and carrier-drug dilution (10 μl of first dilution into 10 ml olive oil to give 10 ng/ml). The natural viscosity of the oil maintained the carrier or carrier-drug combination within the vein graft.

Grafting Procedure

Animals were anesthetized by intraperitoneal administration of ketamine-xylazine (ketamine hydrochloride 5%, 90 mg/kg and xylazine hydrochloride 2%, 5 mg/kg). The procedures were carried out based on the guidelines of the ethics committee of the International Association for the Study of Pain.33 The Urmia University Research Council approved all experiments. A 15-mm segment of the right external jugular vein was harvested on a tube carrier or carrier-drug combination within the vein graft.

Walking track analysis was performed 4, 8, and 12 weeks after surgery based on the work of Bain et al. The distances from the third toe to its heel (PL), the first to the fifth toe (TS), and the second toe to the fourth toe (IT) were measured on the experimental side (E) and the contralateral normal side (N) in each rat. The Sciatic Function Index (SFI) was calculated in each animal by the following formula:

\[
SFI = \frac{(EIT - NIT) + (ETS - NTS) + (EPL - NPL)}{NTS + NPL + 109.5 + 13.3} \times 100
\]

In general, the SFI varies around 0 for normal nerve function, whereas values around –100 SFI represent total dysfunction. In the present study, a value of 0 was considered to represent normal function, and the results in the IOVG/FK506 group were compared to those obtained in the IOVG group. The SFI was a negative value and a higher SFI meant better function of the sciatic nerve.

Muscle Mass

Recovery assessment was also indexed using the weight ratio of the left (injured) to right (uninjured) gastrocnemius muscles 12 weeks after surgery. Immediately after euthanasia, the gastrocnemius muscles were dissected and harvested from intact and injured sides and weighed while still wet, using an electronic balance. All measurements were made by 2 independent observers unaware of the identities of the analyzed groups.

Histological Preparation and Quantitative Morphometric Studies

The operated nerve was dissected from the surrounding tissues, and a segment including several millimeters proximal and distal to the graft was harvested. The middle region of each graft from the animals in the sham-surgery, IOVG, and IOVG/FK506 groups fixed in 2.5% glutaraldehyde. These specimens were cut in 5-μm-thick sections, postfixed in OsO₄ (2%, 2 hours), dehydrated through an ethanol series, and embedded in Epon. Sam-
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Slices were then stained with toluidine blue and examined under light microscopy. Morphometrical analysis was carried out using image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics). Equal opportunity, systematic random sampling, and 2D dissector rules were followed to cope with sampling-related, fiber location–related, and fiber size–related biases.13

Immunohistochemical Analysis

In this study, S100 protein was used as a marker for myelin sheath. Specimens were postfixed with 4% paraformaldehyde for 2 hours and embedded in paraffin. Prior to immunohistochemical analysis, nerve sections were dewaxed and rehydrated in PBS (pH = 7.4). Then the nerve sections were incubated with 0.6% hydrogen peroxide for 30 minutes. To block nonspecific immunoreactions the sections were incubated with normal swine serum (1:50, Dako). Sections were then incubated in anti-S100 antibody solution (1:200, Dako) for 1 hour at room temperature. They were washed 3 times with PBS and incubated in biotinylated anti–mouse rabbit IgG solution for 1 hour. Horseradish peroxidase–labeled secondary antibody solution was applied for 1 hour. All sections were then incubated with 3,3′-diaminobenzidine tetrahydrochloride chromogene substrate solution (DAB, Dako) for 10 minutes. The results of immunohistochemistry were assessed by examination under a light microscope.

Statistical Analysis

Experimental results were expressed as means ± SDs. Statistical analyses were performed using SPSS 18.0 (PASW Statistics). Model assumptions were evaluated by examining the residual plot. Results were analyzed using a factorial ANOVA with 2 between-subjects factors. The Bonferroni test for pairwise comparisons was used to examine the effect of time and treatments. The differences were considered significant when the p value was less than 0.05.

Results

Recovery of Sciatic Nerve Function

Figure 1 shows the mean SFI values in the 3 experimental groups at 4, 8, and 12 weeks after surgery. Prior to surgery, the SFI values in all groups were near zero. After the nerve axotomy, the mean SFI value decreased to −100 due to the complete loss of sciatic nerve function in all animals. Four weeks after surgery, the mean SFI value in the IOVG/FK506 group was −73.3 ± −2.05, compared with −92.8 ± −4.24 in the IOVG group. Eight weeks after surgery, significantly more improvement in SFI was observed in the animals in the IOVG/FK506 group (mean SFI −61.6 ± 3.23) than in the animals in the IOVG group (mean SFI −74.6 ± −3.44; p < 0.05). After 12 weeks, the mean SFI value for the IOVG/FK506 group was −50.8 ± −4.83, an approximate improvement of 48%, in comparison to −64.1 ± −2.0.1 in the IOVG group, an approximate improvement of 34%. Statistical analysis revealed that the recovery of nerve function was significantly faster in the IOVG/FK506 group than in the IOVG group (p < 0.05) and topically administered FK506 promoted functional recovery.

Muscle Mass Measurement

The mean ratios of gastrocnemius muscle weight were calculated. There was a statistically significant difference between the muscle weight ratios of the IOVG/FK506 and IOVG groups (p < 0.05). The results showed that the mean muscle weight ratio of the IOVG/FK506 group was greater than that of the IOVG group and weight loss of the gastrocnemius muscle was ameliorated by topical administration of FK506 (Fig. 2).

Morphological Findings

Table 1 shows the results of quantitative morphometric analyses of regenerated nerves for each of the experimental groups. Four weeks after surgery, the IOVG/FK506 group had significantly more nerve fibers, greater

![Fig. 1. Diagrammatic representation of effects on the SFI. Topical administration of FK506 with vein grafting (IOVG/FK506 group) gave better results in functional recovery of the sciatic nerve than were seen in the animals who underwent vein grafting without FK506 (IOVG group). Data are presented as means ± SDs (error bars). *p < 0.05 versus the IOVG group.](image1)

![Fig. 2. Gastrocnemius muscle measurement. The gastrocnemius muscle was removed from both sides (injured left and intact right) of the animals in the experimental groups and weighed 12 weeks after surgery. Data are presented as means ± SDs. The difference between the left/right ratios in the IOVG and IOVG/FK506 groups was statistically significant. *p < 0.05.](image2)
axon diameter, and greater myelin sheath thickness than the IOVG group (p < 0.05). Although the animals in the IOVG group showed regeneration patterns, the morphometric indices in the IOVG/FK506 group were significantly higher than those in the IOVG group at both 8 and 12 weeks after surgery (Figs. 3–5).

Based on factorial ANOVA analysis with 2 between-subjects factors (group \( \times \) time), the number of nerve fibers and the myelin thickness in the IOVG/FK506 group did not differ significantly between 8 and 12 weeks (p > 0.05). The increase in the mean thickness of the myelin sheath did not show a statistical difference between 8 and 12 weeks within each group (p > 0.05). From Week 8 on, the mean thickness of the myelin sheath did not differ significantly between the IOVG/FK506 and sham-surgery groups (p > 0.05).

**Immunohistochemical Analysis**

Immunoreactivity to S100 protein was extensively observed in the cross-sections of regenerated nerve segments. The expression of S100 protein signal was located mainly in the myelin sheath. The axon also showed a weak expression indicating that a Schwann cell–like phenotype existed around the myelinated axons (Figs. 6 and 7). In both groups, the expression of S100 corresponded with the results of the morphometric analyses.

**Discussion**

The multitude of sciatic nerve injuries and subsequent disabilities provide a strong motivation to develop agents and methods of repair that accelerate axonal regeneration. Entubulation neurorrhaphy is an excellent alternative to short interposition nerve grafts. Vein graft technique is an effective repair method that allows axonal regeneration after short-gap nerve injuries. Systemically administered FK506 has been shown to accelerate axonal regrowth, but the side effects of systemic administration

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**Table 1: Morphometrical analyses of regenerative nerves for each of the 3 experimental groups**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sham Group</th>
<th>IOVG Group</th>
<th>IOVG/FK506 Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 wks</td>
<td>8 wks</td>
<td>12 wks</td>
</tr>
<tr>
<td>no. of fibers</td>
<td>8177 ± 411</td>
<td>8370 ± 446</td>
<td>8379 ± 466</td>
</tr>
<tr>
<td>diameter of fibers (µm)</td>
<td>12.04 ± 0.05</td>
<td>11.93 ± 0.17</td>
<td>12.06 ± 0.33</td>
</tr>
<tr>
<td>diameter of axon (µm)</td>
<td>7.03 ± 0.02</td>
<td>6.97 ± 0.39</td>
<td>6.97 ± 0.46</td>
</tr>
<tr>
<td>thickness of myelin sheath (µm)</td>
<td>2.56 ± 0.01</td>
<td>2.48 ± 0.02</td>
<td>2.56 ± 0.01</td>
</tr>
</tbody>
</table>

* Significantly different from the sham-surgery group (p < 0.05).

**Fig. 3.** Line graph showing the quantitative results of fiber counting. The mean number of nerve fibers in the sham-surgery group was nearly 8177 ± 411. Both the IOVG group and the IOVG/FK506 group had fewer fibers than the sham-surgery group, even at the end of the study. From 4 to 8 weeks, the IOVG/FK506 group had significantly more nerve fibers than the IOVG group (p < 0.05), and this difference increased (in favor of IOVG/FK506 group) at the end of the study period.
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of this agent can be a major drawback. Thus, formulating a topical dosage that mimics the same neurotrophic stimulus with less risk of systemic toxicity would be desirable.

Selection of an appropriate method for evaluating functional recovery of nerve regeneration is extremely important. Walking is a coordinated activity involving sensory input, motor response, and cortical integration. Therefore, walking track analysis (SFI) is a comprehensive test. The results of the present study showed that application of FK506 in a vein graft resulted in faster and significantly improved functional recovery of the sciatic nerve.

A 12-week experimental period was chosen for evaluation of regeneration process because functional recovery after repair of a transected peripheral nerve in rats occurs during this period of time. It is not clear, however, whether functional recovery of the sciatic nerve would have continued if our study had gone further. Longer-term study periods are needed to investigate whether topical application of FK506 can have more beneficial effects on functional recovery of peripheral nerve.

As the posterior tibial branch of the sciatic nerve regenerates into the gastrocnemius muscle, it will regain its mass proportional to the amount of axonal reinnervation. In the present study, both experimental groups showed reduction in muscle mass in the left (injured) leg relative to the right (uninjured) leg at 12 weeks after surgery, but the mean left/right gastrocnemius muscle weight ratio was significantly greater in the IOVG/FK506 group than in the IOVG group, indicating indirect evidence of successful end organ reinnervation in the FK506-treated animals.

In the histological studies, quantitative morphometrical indices of regenerated nerve fibers showed significant differences between the IOVG and IOVG/FK506 groups, indicating a beneficial effect of topical application of FK506 on the nerve regeneration. With respect to myelin thickness there were no significant differences between IOVG/FK506 and that of the sham-surgery group from Week 8 to the end of the study period. Other investigators studying the effect of local administration of FK506 in a rat sciatic nerve model over an 8-week study period have reported that the regenerated myelinated fibers showed a more mature morphometric profile at 8 weeks.

In the immunohistochemical analysis, the expression of axon and myelin sheath special proteins was evident in both groups, indicating normal histological structure. Specimens from the IOVG/FK506 group, however, were more strongly positive for S100 immunoreactivity, which provided further evidence of the presence of both regen-
A major drawback to the use of immunosuppressive agents is their side effects. Weight loss accompanied by diarrhea is known to be associated with systemically administered FK506 in rats. To overcome this drawback, authors have suggested minimizing the dosage or limiting the duration of therapy.

The ability of FK506 to increase nerve regeneration in vivo is reported to be dose-dependent, with the best results of systemic administration obtained with a daily dose of 5 mg/kg in the rat. Thus, we decided to test whether topical administration of FK506 would result in comparable improvement in nerve regeneration through a tubulized 10-mm gap in sciatic nerve transection models. Results of the use of FK506 in peripheral nerve regeneration differ in the literature. One possible explanation for the relative variability of the results of studies of experimental nerve injuries is the variety of models and testing methods used. It has been reported that FK506 administered at a low dose (1 mg/kg per day intraperitoneally) accelerated recovery of the walking track pattern by about 1 week with respect to control rats. Other authors also reported that the histomorphometric analysis performed at 7 weeks after grafting showed greater myelinated fiber density in FK506-treated animals. Similarly, a higher axonal count was found in FK506-treated rats (0.3 or 0.6 mg/kg subcutaneously) than in untreated animals 2 weeks after autograft repair. Improvement in the rate of axonal regeneration by FK506 is likely due to a direct effect on the axotomized neurons that speeds up elongation.

Furthermore, additional action to hasten Wallerian degeneration should be taken into consideration. The Schwann cell and its basal lamina are vital components in the environment in which regenerating axons extend to grasp their peripheral targets. Schwann cells from the distal stump of a transected nerve start proliferating, help inflammatory infiltrating cells to eliminate debris, and upregulate synthesis of trophic factors throughout Wallerian degeneration, and regeneration is failed or delayed in situations where the process of Wallerian degeneration is diminished. FK506 has been reported to stimulate proliferation of Schwann cells cultured from predegenerated nerves and to decrease the myelin debris found in the distal stump of transected nerve initially after surgery. It has also been reported that FK506 did not affect the amount of macrophages infiltrating injured nerve tissue.

Stimulating effects of FK506 on peripheral nerve regeneration have been reported based on molecular mechanisms. Binding to FKBP-12 and inhibition of calcineurin increases the phosphorylation of several substrate molecules like GAP-43, which is a protein that plays an important role in neural plasticity and is highly expressed in the regenerative growth cones. Nonimmunosuppressant derivatives of FK506 do not inhibit calcineurin but accelerate nerve regeneration.

However, a topical formulation that provides the same neurotrophic stimulus would be ideal because of less systemic toxicity. Entubulation neurorrhaphy using a FK506-loaded vein graft as an in situ FK506 delivery system in bridging the defects could be considered as an excellent alternative to short-interposition nerve grafts. The use of a vein graft seems to have several distinct advantages for the treatment of transected peripheral nerves: 1) it can be used as an autogenous transplant; 2) it does not provoke any noticeable foreign-body reaction; 3) it can be harvested by minor surgery with minimal risk.
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of complications; and 4) no functional deficit or injury occurs at the donor site in contrast to the situation with nerve and artery grafts. Moreover, with the use of an interposition graft, the regenerating fibers are forced to grow through scar tissue, which often results in decreased efficacy of repair and aberrant facial reinnervation.

Conclusions

In the present study, FK506 applied topically at the time of sciatic nerve repair using vein graft neurorrhaphy demonstrated promising results in nerve regeneration. Thus, dose-response studies should be conducted for FK506 to determine the combination of graft and compound that achieves maximal efficacy in nerve transection models.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Mohammadi. Acquisition of data: Mohammadi, Fallah. Drafting the article: Mohammadi. Critically revising the article: all authors. Reviewed submitted version of manuscript: Amini. Administrative/technical/material support: Amini. Study supervision: Azizi.

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