Does FK506 reduce the size of the watershed area after vascular injury of the sciatic nerve?

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Abstract
Aim: FK506 (also known Tacrolimus, Prograf) is an immunosuppressant drug which is used to prevent rejection after organ transplantation. Although there are several studies on neuroprotective effect of FK506 on brain ischemia, few reports on effects of FK506 after peripheral nerve ischemia have been reported. In the present study, we examined the size of watershed area after stripping of the epiureial vessels and studied the effect of FK506 on reduction of the size of watershed area. Materials and Methods: Forty-eight adult female rats were used and randomly divided into four groups as control, sham, FK506-treated and vehicle-treated. In FK506-treated and vehicle-treated groups epiureial vessels around the sciatic nerve (vasa nervorum) were stripped. Additionally, FK506-treated group were received subcutaneous injection of 5 mg/kg FK506. Percent of watershed area (100 × total watershed areas / total nerve area) after stripping and FK506 treatment was calculated. Results: We found no significant difference in comparison of the total size of watershed areas in FK506 and vehicle-treated groups or even the percent of the watershed area in both groups. Conclusions: We think that this study will be helpful to understand neuroprotective effect of FK506 and will give an insight into sparing of the nerve fibers from vascular injuries of the peripheral nerve.

Keywords: watershed area, epiureial vessels, FK506, sciatic nerve, vasa nervorum.

Introduction
FK506 (also known Tacrolimus, Prograf) is an immunosuppressant drug isolated from Streptomyces tsukubaensis in Japan [1]. In clinical practice, it is used to prevent rejection after organ transplantation [2–4]. FK506 is known to inhibit calcineurin-mediated T-cell activation by forming a complex with FKBP12 [5]. FK506 have a potential neuroprotective effect and ameliorate apoptotic cell death, which also play an important role in ischemic cell death [1, 3]. Neuroprotective effect of FK506 was first demonstrated to be mediated by inhibition of calcineurin activity [6]. Mechanisms for anti-ischemic [7], antiapoptotic [8, 9] and anti-inflammatory [7] effects of FK506 have also been studied.

FK506 protects against ischemic damage in several models of focal and global ischemia [10]. In the literature, there are several studies on neuroprotective effect of FK506 on brain ischemia [6, 11, 12]. However, there are few reports on effects of FK506 after peripheral nerve ischemia [13]. In a recent study, we examined effect of FK506 on functional recovery after focal ischemia of the sciatic nerve [14]. We produced the focal ischemia by stripping of the epiureial vessel around the sciatic nerve and found beneficial effect of FK506 on the superperineural degeneration. We suggested that daily 5 mg/kg administration of FK506 enhance remyelination and accelerate functional recovery. In the present study, we examined the size of watershed area after stripping of the epiureial vessels and studied the effect of FK506 if it reduces the size of watershed area.

Materials and Methods
Animals
The present study was performed on 48 female Wistar rats with body weights ranging from 200–250 g. They were divided randomly into four groups as control, sham, FK506-treated and vehicle-treated. All animals were housed in plastic containers lined with wood shavings and maintained on a 12-hours light-dark cycle. Standard rat chow and water were provided ad libitum. All experiments were performed in accordance with the international standards for animal experimentation and following approval by our local institution’s Animal Care and Ethics Committee.

Surgery
On the day of the experiment, each rat was weighted and then anesthetized with an intramuscular injection of Xylazine HCl (15 mg/kg) and Ketamine (100 mg/kg). Left side of the hind limb was shaved and swabbed with antiseptic solution. One longitudinal skin incision was applied in the outer side of the thigh. A hind limb muscle splitting approach was used to expose the left side sciatic nerve and its three branches under magnification with a fiber-optic illuminated operating microscope (Olympus SZ61). Careful blunt dissection was performed to isolate the sciatic nerve from the
surrounding connective tissue over a length of 3 to 3.5 cm. Epineurial vessels around the sciatic nerve (*vasa nervorum*) were then stripped for 2–2.5 cm as previously described [15]. Two fine sutures were tied to the adjacent muscle to mark the borders of the stripped area. The wound was closed with a 4–0 Ethilon® suture and rats were allowed to recover in a postoperative room.

**FK506 administration**

FK506-treated group were received subcutaneous injection of 5mg/kg FK506 (Prograf, Eczacibasi, Istanbul). FK506 administration was given for four weeks and was not interrupted (including weekends) during postoperative days. First injection was performed from the day of epineurial stripping to day of animal sacrifice. The same volume of saline was administered to the vehicle-treated animals.

**Tissue sampling and preparation**

Four weeks after induction of the stripping of the epineurial vessels, the animals were administered an overdose of chloral hydrate intraperitoneally. The sciatic nerve was re-exposed and the stripped area of the nerve was sampled. Samples were fixed with 4% glutaraldehyde in 0.1 M Sorensen’s phosphate buffer solution (pH 7.3) for two hours and then post-fixed with 2% osmium tetroxide in the same buffered solution for one hour. After dehydration through a graded series of ethanol, samples were embedded in epoxy resin (Araldite CY212, Agar Scientific Ltd., Stansted, UK). Transverse semi-thin sections (1 µm) were stained with Toluidine Blue and were examined and photographed with a light microscope (Olympus CX41) equipped with a video camera.

**Morphometric analysis**

Morphometric analysis of sections was performed by using a light microscope (Carl Zeiss Axioskop 2) equipped with a video camera (Carl Zeiss Axiocam) connected to an image analyzer system (Axiovision Release 4.2). After defining and adjusting the magnification (10× or 20×) of the microscope and taking the images, watershed areas and total nerve area were marked manually and calculated automatically by the analyzing system as square micrometer. Percent of watershed area (100 × total watershed areas / total nerve area) was calculated for each group.

**Data analysis**

For any given parameter, data from all experimental groups were tested using one-way analysis of variance (one-way ANOVA) and with *post hoc* Tukey’s test. A *p*-value less than 0.05 considered as significant. For analysis, GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, Calif) was used.

**Results**

Immediately after stripping of the epineurial vessels, the stripped area of the sciatic nerve became very edematous. It was observed in all animals that the epineurium around the sciatic nerve was not damaged during epineurial stripping.

Control and sham-operated groups did not show watershed areas when the sections were examined by light microscopy. In FK506 and vehicle-treated groups watershed areas were located in the subperineurial area of the nerve (Figure 1). In some sections, the watershed areas had a fascicular localization and whole fascicle was affected from anoxia and therefore showed degeneration (Figure 1b). Additionally, in some instances watershed areas were observed in neighboring fascicles of the sciatic nerve (Figure 2). Numerous damaged myelin residues were observed in the vehicle and FK506-treated groups. The amount of myelin debris was higher in the vehicle-treated group than those in the FK506-treated group.

![Figure 1](image1.png) **Figure 1** – Micrograph showing three watershed areas located in the subperineurial area of the posterior tibial nerve. A, C, D: Watershed areas in the subperineurial area of the nerve. B: Fascicular localization of the watershed area. Scale bar: 200 µm.

![Figure 2](image2.png) **Figure 2** – Micrograph showing watershed areas in the three neighboring fascicles of the sciatic nerve. PTN: Posterior tibial nerve, PN: Peroneal nerve, SN: Sural nerve. Scale bar: 400 µm.

After the epineurial stripping, watershed areas and total nerve areas were measured. Comparison of the total nerve areas revealed that there was no statistical significance between all groups of the study. Total nerve area of FK506-treated and vehicle-treated groups were determined as 909582±189941 µm², 901681±122587 µm², respectively. Percent of watershed area in FK506-treated and vehicle-treated groups was 25.7±13.1%, 26.5±11.4%, respectively (Figure 3).
No significant difference (p>0.05) was observed in comparison of the total size of watershed areas in FK506-treated and vehicle-treated groups or even the percent of the watershed area in both groups.

![Graph showing percent of watershed area in FK506 and vehicle-treated groups.](image)

**Figure 3 – Percent of the watershed area in FK506 and vehicle-treated groups.**

**Discussion**

It has been suggested that peripheral nerve has a marked resistance to ischemic changes [16]. Additionally, peripheral nerve is assumed to be relatively resistant because of its low energy needs [17–19], extensive anastomoses between endoneurial and extraneurals vessels [20, 21], and ability to survive on anaerobic conditions [18]. However, peripheral nerve ischemia is common, occurring in the ischemic neuropathies associated with common disorders such as diabetes, collagen vascular disorders, and thromboembolic disease [22].

FK506 has been studied in terms of its neuroprotective, anti-ischemic effects, anti-inflammatory, and antiapoptotic actions [23]. Although most of the experimental data are derived from trauma such as crush and cut injuries of the peripheral nerve, the neuroprotective potential of FK506 and Cyclosporin A has also been reported in brain ischemia [24]. However, the mechanism by which FK506 prevents ischemic brain damage is still unknown. Although there are many reports on neuroprotection in brain ischemia [3, 12, 23, 25], there are few reports on potential neuroprotective effects of FK506 in peripheral nerve ischemia [13, 14]. Using microspheres injections Kihara M et al. [13] studied effect of two doses of FK506 in protecting peripheral nerve from ischemic fiber degeneration. They produced ischemia by injecting microspheres into the left femoral, hypogastric, and superior gluteal arteries. After embolization, they administrated different dosages of FK506 into the same arteries. They suggested that small dose of FK506 protects the sciatic nerve from ischemic fiber degeneration. In a recent study, we found that administration of the FK506 produced a neuroprotective role after stripping of the epineurial vessels. The shape and appearance of the myelin sheaths were comparatively in a good form in FK506-treated group than those of vehicle-treated group.

Neuroprotective effects of FK506 against ischemic damage in focal and global ischemia have been documented in the literature [10, 23]. Furuichi Y et al. [23] administered single dose of FK506 intravenously after middle cerebral artery occlusion. They reported that FK506 reduces brain infarction size and also ameliorates long-term neurologic deficits. Additionally, therapeutic time window for the FK506 administration has also been proposed [3, 10]. Takamatsu H et al. [10] reported that FK506 showed powerful neuroprotective effects against cortical ischemic damage, and its therapeutic time window was at least three hours after the onset of stroke. Using transient ischemia model by ligation of the middle cerebral artery, Arii T et al. [3] administrated FK506 to decrease the damage and found that FK506 reduced the infarct size in cortex when given at 30 or 60 minutes after induction of ischemia, but not at 120 minutes. In the present study, we evaluated the infarct or watershed areas of the sciatic nerve after stripping of the epineurial vessels and found that FK506 did not have an effect on reduction of the infarct size. No significant difference (p>0.05) was observed in comparison of the total watershed area in FK506-treated and vehicle-treated groups or even the percents of the watershed area in both groups. However, in a recent study we showed its role to enhance the quality of the myelination in the watershed area. In the literature it has not, to our knowledge, been reported the potential of the FK506 treatment on reduction of the side of watershed areas in sciatic nerve after ischemic or devascularization injury.

In clinical practice, the diagnosis and treatment of patients with peripheral nerve injuries are often delayed [26]. Although we started FK506 administration at the first postoperative day, such therapeutic time window might be existed in the focal ischemia of the sciatic nerve produced by stripping of the epineurial vessels. We think that further studies should be performed to verify this probability by studying therapeutic time window of FK506 in vascular injuries of the peripheral nerve.

Peripheral nerve undergoes fiber degeneration when subjected to ischemia. A common pattern of centrifascicular degeneration with subperineurial sparing of nerve fibers may be found in necrotizing angiopathy and following nerve grafting [27, 28]. The mechanism of the centrifascicular degeneration with subperineurial sparing of nerve fibers pattern is not known but some hypotheses have also been proposed [17]. According to one hypothesis subperineurial fibers might be spared because oxygen diffuses from surrounding viable tissues partway into nerve. Earlier studies suggested that sufficient oxygen could diffuse into peripheral nerve from its environment to delay ischemic conduction failure and to prevent fiber degeneration in ischemic nerves [17, 29, 30]. In the present study, FK506 administration did not effect on reduction of the percent of watershed areas although all watershed areas have been observed in the subperineurial area of the sciatic nerve. We think that this hypothesis may not be valid and should be tested by further studies.

**Conclusions**

In the literature it has not, to our knowledge, been reported the potential of the FK506 treatment on reduction of the percent of watershed area in sciatic nerve after ischemic or devascularization injury.
We think that this study will be helpful for neuroscientist who dealing with peripheral nerve injury.

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References

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