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*Original Investigation*

# Topical Application of Cyclosporine Reduces Epineurial Fibrosis: Gross Post-surgical, Histopathological and Ultrastructural Analysis in a Rat Sciatic Nerve Model

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## ABSTRACT

**AIM:** To investigate the anti-scarring potential of topical cyclosporine on rat sciatic nerves.

**MATERIAL and METHODS:** Both sciatic nerves were exposed in 24 adult male albino Wistar rats, and an abrasion injury was made on the biceps femoris close to the sciatic nerve. Cotton pads soaked with cyclosporine (5 mg/mL) and saline (0.9% NaCl) were placed around the nerves for 10 minutes in the experimental group and control group, respectively. All rats were sacrificed 8 weeks later and the sciatic nerves were examined. Epineurial adhesions were assessed using light and electron microscopy. Quantitative histological parameters, epineurial thickness, and scar density were evaluated in the histological investigation.

**RESULTS:** Significantly fewer epineurial adhesions were observed in the cyclosporine group in the post-surgical assessment, and the histopathological and ultrastructural examinations of the nerve segments than in the controls. The cyclosporine-treated animals had a statistically significant reduction in the density and quantity of epineurial scarring compared with the controls.

**CONCLUSION:** Topical cyclosporine effectively reduces epineurial scar formation on rat sciatic nerves.

**KEYWORDS:** Cyclosporine, Sciatic nerve, Epineurial fibrosis, Rat

## INTRODUCTION

Epineurial fibrosis is the leading cause of unsatisfactory results in peripheral nerve surgery. Adhesions formed by collagen fibers cause tethering, which impedes the flexibility of the nerve during limb movement. As a result, traction and compression of the nerve may lead to ischemia and loss of function as well as persistent pain and diminished sensation (8,12,26). Numerous efforts, both surgical and pharmacological, have been made to prevent epineurial fibrosis including nerve transposition, vein wrapping, bioabsorbable physical barriers, and grafts and flaps, all with suboptimal outcomes (2,7,10,14,25). Therefore, any novel technique or pharmacological agent for the purpose of extraneural scar reduction will increase the success rates of peripheral nerve surgery. In the literature, tacrolimus has been shown to be effective in promoting nerve regeneration by reducing epineurial scar formation (9,16,28).

We investigated the potential of topically applied cyclosporine, an immunosuppressive agent, in epineurial scar inhibition on rat sciatic nerve injury. Cyclosporine is a well-known

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immunosuppressive agent used in organ transplantation and for the treatment of ocular inflammatory diseases in ophthalmology; however it has not been well studied in peripheral nerve pathologies to date (21). We believe that this is the first study to investigate the anti-scarring effect of cyclosporine on peripheral nerve ultrastructurally and in gross post-surgical and histopathological analysis.

## MATERIAL and METHODS

Twenty-four adult male albino Wistar rats that weighed 250-300 g were housed in a humidity- and temperature-controlled environment (21±3°C and 65±5%, respectively) with a 12-24h light-dark cycle. The rats were given standard rat chow and tap water *ad libitum*. Ethical approval was obtained from the local ethics committee of Gulhane Military Medical Academy (Ankara, Turkey) prior to experiments.

### Surgical Technique

Ketamine hydrochloride (40mg/kg) and xylazine (5mg/kg) were used for intramuscular general anesthesia. Both sciatic nerves were exposed and separated from the adjacent tissue and the tibial-peroneal branches were dissected through the sciatic foramen. An epineurial abrasion injury was made on the biceps femoris muscle with a nylon brush while retracting and protecting the sciatic nerves and all branches. The animals were allocated into, the control and the experiment (cyclosporine) groups, each with 12 animals. Cyclosporine (5mg/mL) and saline (0.9% NaCl)-soaked cotton pads (5x10 mm) were placed around the nerves for 10 minutes in the experiment group and control group, respectively. The nerves were irrigated after the removal of the pads. The wounds were closed taking care to maintain anatomical integrity. No postoperative complications (mortality or neurological deficits) were observed in either group.

The rats were examined daily in the postoperative period for surgical wound healing and weekly for neurological functions, specifically any change in foot posture, toe spreading, plantar and dorsal flexion was noted.

### Postoperative Gross Anatomical Evaluation

Eight weeks after the first surgical procedure, the neurolysis site around the sciatic nerves of each rat was meticulously dissected under deep ether anesthesia. The dissecting surgeon was blinded to the group allocation. Epineurial adhesions were assessed based on observations of the right sciatic nerves from both study groups (n=24) and graded using the numeric protocol of Petersen et al. (Table I) (15).

### Histological and Ultrastructural Analysis

Tissue samples were prepared, sectioned, stained and examined using the method described previously by Albayrak et al. (1). A senior histologist (N. D.), who was blinded to group allocation, performed all histological and ultrastructural examinations and analyses.

### Statistical Analysis

Statistical analysis was performed using the statistical package SPSS v20.0. Comparisons were applied using the student t test or one-way ANOVA. Mann-Whitney U test or Kruskal-Wallis test was used when variables were not normally distributed. The categorical variables between the groups were analyzed by using the Chi-square test or Fisher's exact test.

## RESULTS

### Clinical Follow-up

There was no significant difference in the wound healing characteristics or neurological functions between the treatment (cyclosporine) group and the control group ($p>0.05$).

### Gross Post-surgical Results

All rats were anesthetized and sacrificed after 8 weeks and the sutures were removed. No inflammation or surgical site infections were detected. All cyclosporine-treated nerve segments had significantly fewer adhesions and could be dissected more easily than those in the control group (Figure 1A, B). Skin, muscle, and deep fascia incisions were healed completely in all groups as established using the numeric grading scheme of Petersen et al. ($p>0.05$) (15). The cyclosporine-treated group had significantly fewer perineurial adhesion and better separability than the control group ($p<0.0001$). In terms of nerve adherence and separability, no significant difference was found between the sciatic nerves of both extremities in the treatment (cyclosporine) and control gorups ($p>0.05$). Table II shows the gross anatomical evaluation findings of the right sciatic nerves.

### Histopathological Analysis

The control group's nerve segments were surrounded by significant fibrous tissue comprising collagen fibers; the

**Table I:** Numerical Grading Scheme Described for Gross Evaluation by Petersen et al. (15)

| Tissue | Grade | Definition |
|---|---|---|
| Skin and Muscle Fascia | 1 | Skin or muscle fascia entirely closed |
| | 2 | Skin or muscle fascia partially open |
| | 3 | Skin or muscle fascia completely open |
| Nerve Adherence and nerve separability | 1 | No dissection or only mild blunt dissection |
| | 2 | Some vigorous blunt dissection |
| | 3 | Sharp dissection required |

epineurium thickness (cross-sectional measurement) of all nerves of the controls was greater than in the cyclosporine-treated group (Figure 2A, B). In addition, the collagen fibers of the epineurium in the cyclosporine group were much looser with a lower number of fibroblasts than the controls. Quantitatively, the epineurial thickness and scar density were significantly higher in the control group compared with the treatment (cyclosporine) group based on left sciatic nerve measurements (Tables III, IV).

### Ultrastructural Analysis

In the ultrastructural examinations, thinner collagen fibers were observed in the cyclosporine-treated nerve segments than in the controls. Moreover, the control group animals

**Table II:** Gross Post-surgical Epineurial Scarring Scores

| | | Control | | Cyclosporine | |
|---|---|---|---|---|---|
| | | n | % | n | % |
| Skin | 1 | 12 | (100.0) | 12 | (100.0) |
| Muscle-Fascia | 1 | 9 | (75.0) | 8 | (66.7) |
| | 2 | 3 | (25.0) | 4 | (33.3) |
| p | | | 0.877 | | |
| Nerve Adherence* | 1 | 0 | (.0) | 9 | (75.0) |
| | 2 | 7 | (58.3) | 3 | (25.0) |
| | 3 | 5 | (41.7) | 0 | (.0) |
| p | | | 0.0001 | | |
| Separability* | 1 | 0 | (.0) | 10 | (83.3) |
| | 2 | 4 | (33.3) | 2 | (16.7) |
| | 3 | 8 | (66.7) | 0 | (.0) |
| p | | | 0.0001 | | |

**Nerve tissue adherence and separability were significantly better in the Cyclosporine-treated group than in controls ($p < 0.0001$).*

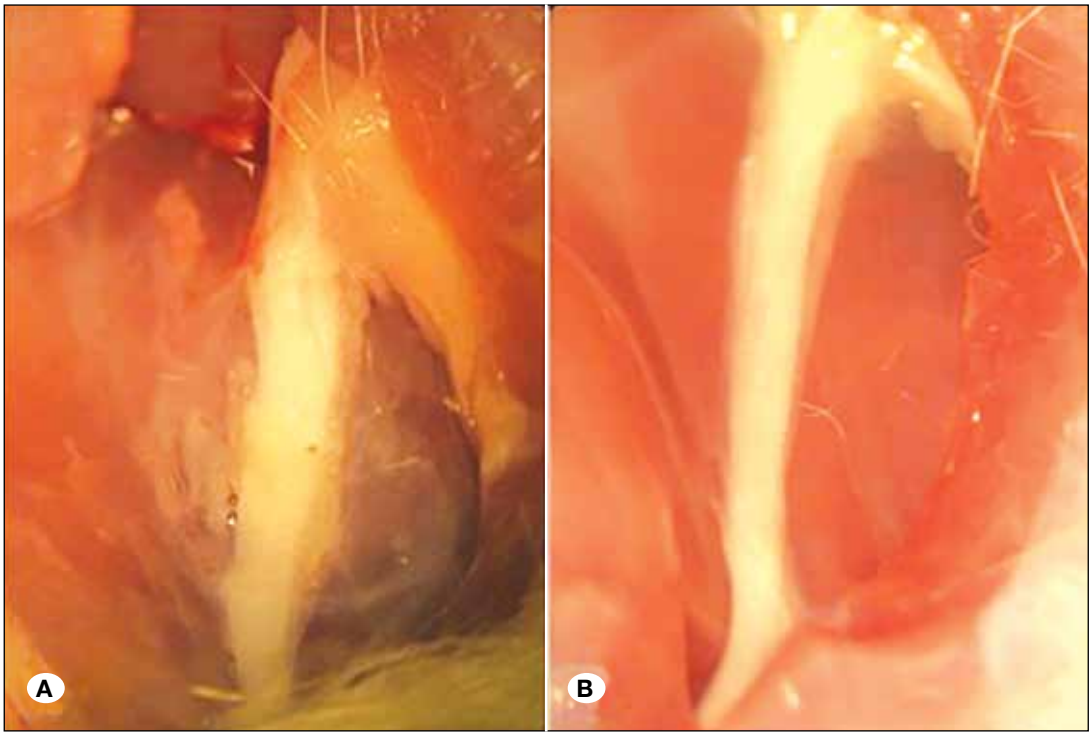


**Figure 1:** Photographs of the sciatic nerves at 8 weeks post-surgery. **A)** Sciatic nerves treated with saline (0.9% NaCl) were surrounded by diffuse scaring and tethered to the surrounding tissue. Peroneal and tibial components could not be separated with blunt dissection. **B)** The cyclosporine-treated nerves were easily dissected from the surrounding tissue.

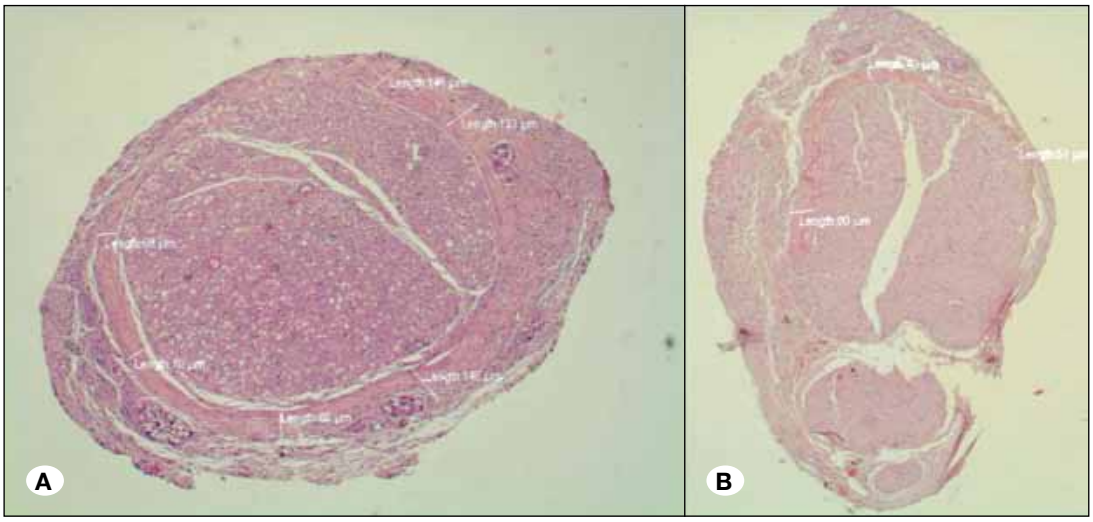


**Figure 2:** Photomicrographs of cross-sections of nerve segments. **A)** The control group; **B)** cyclosporine-treated group. Thickness of the epineurium of sciatic nerve segments treated with cyclosporine was significantly less than the segments from nerves treated with saline only.

**Table III:** Epineurial Scar Tissue Formation Index

| Sciatic Nerve Segment | Control | Cyclosporine |
|---|---|---|
| 1 | 91 | 50 |
| 2 | 152 | 54 |
| 3 | 97 | 40 |
| 4 | 90 | 68 |
| 5 | 124 | 24 |
| 6 | 162 | 30 |
| 7 | 176 | 35 |
| 8 | 209 | 47 |
| 9 | 144 | 61 |
| 10 | 78 | 42 |
| 11 | 109 | 45 |
| 12 | 106 | 38 |
| | 128.17±40.44 (117) | 44.50±12.58 (44) |
| p | **0.0001** | |

*Epineurial thickness of the control group is significantly higher than the cyclosporine group demonstrating more scar tissue formation (p<0.0001).*

**Table IV:** Epineurial Scar Density*

| Sciatic Nerve Segment | Control | Cyclosporine |
|---|---|---|
| 1 | 2.33 | 1.15 |
| 2 | 2.45 | 1.75 |
| 3 | 4.00 | 1.59 |
| 4 | 3.56 | 0.86 |
| 5 | 3.60 | 2.2 |
| 6 | 4.30 | 1.15 |
| 7 | 5.50 | 0.9 |
| 8 | 4.20 | 2.10 |
| 9 | 3.20 | 1.8 |
| 10 | 2.00 | 2.2 |
| 11 | 4.25 | 1.12 |
| 12 | 3.40 | 2.18 |
| | 3.57±0.99 (3.58) | 1.58±0.53 (1.67) |
| p | **0.0001** | |

**Fibroblast/ fibrocyte ratio. p<0.0001.*

demonstrated fewer fibrocytes and more activated fibroblasts with granulated cytoplasms (Figure 3A, B).

## DISCUSSION

Perineurial fibrosis has long been an unresolved issue in peripheral nerve surgery. Although many clinical attempts including nerve wrapping, microsurgical neurolysis, bioabsorbable gels, low-dose radiotherapy, physical barriers, and chemical agents such as aprotinin, mitomycin C, human amniotic fluids have been tried, none have provided satisfactory results in epineurial fibrosis prevention (4,5,7,10,11,14,19,25). Surgical procedures result in mechanical injury thus triggering the wound healing process. There are two distinct phases in the repair process: a regenerative phase, in which damaged cells are replaced by cells of the same type, and the fibrosis phase, which results in collagen deposition through which normal tissue is replaced by permanent scar tissue (27). Fibroblasts are the key mediator of this process. Fibroblast proliferation leads to abnormal deposition of collagen type I, which results in scar formation. Accordingly, we hypothesized that suppression of fibroblast activation through topically applied cyclosporine,

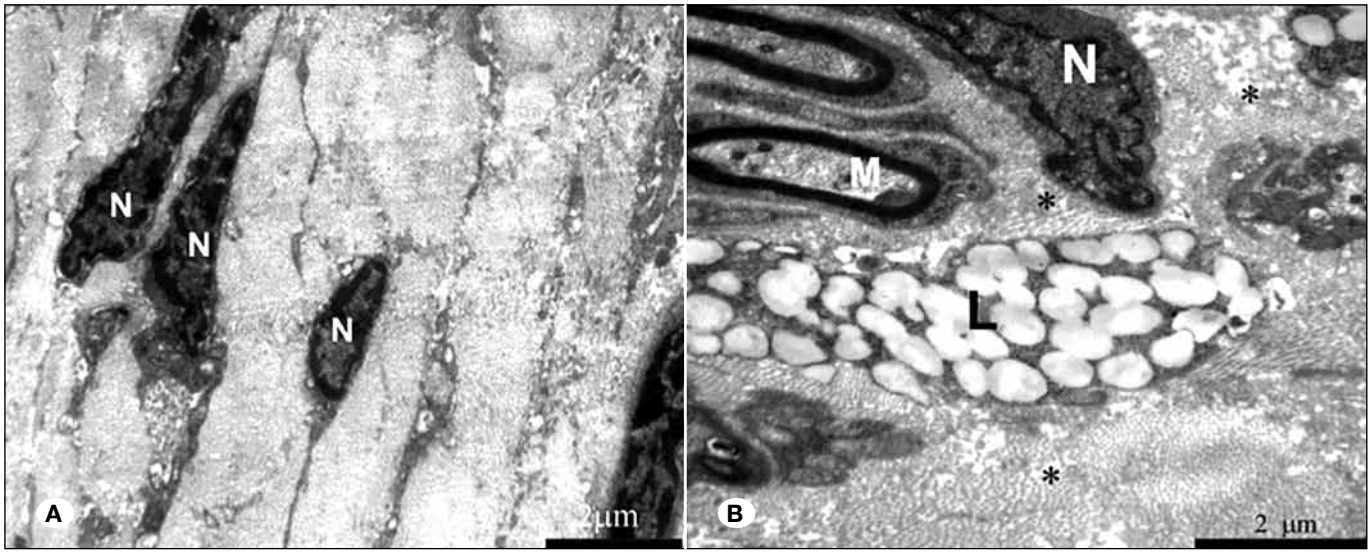


**Figure 3:** Electron microscopic images of sections from cyclosporine-treated **(A**), and saline-treated **(B)** nerves. Examination demonstrates that the cyclosporine-treated nerves contained less activated fibroblasts with narrow and elongated cytoplasms and looser collagen fibers than the control group. In the control group, dense collagen fibers spreading between the nerve fibers damage the reticular structure. Lipid granules compressing the myelinated nerve fibers can be seen (**L:** Lipid, **N:** Nucleus, **M:** Myelinated nerve fiber).

an immunosuppressant agent, would positively impact on postoperative perineurial fibrosis. In a recent study, Que et al. showed that tacrolimus, another immunosuppressant agent, reduced scar formation in a rat sciatic nerve transection-anastomosis model (16). They investigated the systemic effect of tacrolimus 6 weeks after surgery based on histopathological and functional analysis. We investigated the effects of cyclosporine on sciatic nerves of rats 8 weeks after surgery by examining the nerve segments ultrastructurally using electron microscopy (EM) to assess the anti-scarring potential of cyclosporine.

Cyclosporine is a calcineurin inhibitor, a calcium-dependent intracellular signaling protein, which binds to cyclophilin receptors. Several signalling pathways activate in order to increase the intracellular calcium concentration when T-cells are stimulated by an antigen in the normal physiologic process. As a result, increased calcium activates calcineurin, which is a calcium/calmodulin-dependent serine threonine protein phosphatase. Activated calcineurin dephosphorylates nuclear factor of activated T-lymphocytes (NFATs) (23). The biochemical effect of calcineurin inhibitors, such as cyclosporine and tacrolimus, is ultimately the inhibition of T-cell activation and overall immune response (22). This inhibition process also counteracts the release of T-cell derived lymphokines, including IL-2, IL-3, IL-4 and gamma interferon (27). IL-4 in particular is known to be a potent profibrotic mediator. Human fibroblast subtypes have receptors for IL-4 and in vitro studies showed that stimulation of IL-4 induced the synthesis of the extracellular matrix proteins, types I and III collagen and fibronectin constitute the underlying mechanism of scar formation (3,20). In this context, inhibition of this lymphokine may be a contributory factor to antiscarring effect of calcineurin inhibitors. Another mechanism on reducing scar formation seems to be the suppression of fibroblast proliferation. Que et al. demonstrated that gastric lavage of tacrolimus induces fibroblast apoptosis, which contributes to the suppression of fibroblast proliferation ending up scar formation reduction in rats (17). Although our study did not aim to study fibroblast apoptosis, it may be the underlying mechanism of the anti-scarring effect of cyclosporine in a similar fashion, as being a counterpart of tacrolimus. In the aforementioned study, tacrolimus was applied by enteral route in a nerve transection model, in contrast we topically applied cyclosporine on the exposed nerves.

The most common adverse effects of systemic cyclosporine is acute and chronic nephrotoxicity, hepatotoxicity, and cardiotoxicity by the generation of reactive oxygen species and lipid peroxidation (13,18). Furthermore cyclosporine has been used effectively in ophthalmology as a topical agent for many years (24). The anti-scarring potential of other topically-applied pharmacologic agents on peripheral nerves have been reported in the literature (1,4,6,25). Considering these factors, topical application of cyclosporine rather than enteral administration seemed to be adequate for our experiment. Although our literature search revealed no studies that have addressed the potential neurotoxic effects of cyclosporine on intact epineurium, we observed no neurologic hindlimb deficits in our rats during the 8 weeks. This may be due to the barrier function of intact epineurium. There were no histopathological or ultrastructural signs of damage to the epineurium. Our results showed that a single 5 mg/mL topical application of cyclosporine was effective at decreasing epineurial scar formation without negative neurological function impacting or wound healing. There were significant improvements in nerve separability and adherence in the Petersen scores and the scar tissue formation index, and the scar density scores were significantly lower in the treatment (cyclosporine) group compared with the controls. The quantitative histological parameters were in agreement with the anatomical observations. EM findings also showed that the cyclosporine-treated nerves had thinner

and looser collagen fibrils with less fibroblast activation (thinner and elongated cytoplasms) than the saline-treated nerves. The structure of the epineurium was damaged in the control group. These findings show that fibroblast activation and collagen synthesis were significantly lower in cyclosporine-treated nerves than in controls; topical application of cyclosporine effectively suppressed fibroblast activation. However, further studies are needed to elucidate the exact mode of action of cyclosporin on fibroblasts.

## CONCLUSION

Topically-applied cyclosporine was effective in preventing postoperative epineurial fibrosis on rat sciatic nerves after peripheral nerve surgery with no adverse effects. Novel and well-planned studies are needed to translate the experimental information as obtained from our study into clinically effective methods, because there are many potential targets and strategies in preventing epineurial fibrosis.

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