



Functional, Histopathological and Immunohistochemical Assessments of Cyclosporine A on Sciatic Nerve Regeneration Using Allografts: A Rat Sciatic Nerve Model

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ABSTRACT

Objectives: To study the functional, histopathological and immunohistochemical effect of cyclosporine A on sciatic nerve regeneration using allografts in a rat sciatic nerve model.

Methods: Thirty male white Wistar rats were divided into three experimental groups (n=10), randomly: Normal control group (NC), allograft group (ALLO), CsA treated group (ALLO/ CsA). In NC group left sciatic nerve was exposed through a gluteal muscle incision and after homeostasis muscle was sutured. In the ALLO group the left sciatic nerve was exposed through a gluteal muscle incision and transected proximal to the tibio-peroneal bifurcation where a 10 mm segment was excised. The same procedure was performed in the ALLO/ CsA group and the animals were treated with interaperitoneal administration of cyclosporine A. The harvested nerves of the rats of ALLO group were served as allograft for ALLO/ CsA group and vice versa. The NC and ALLO groups received 300 µL sterile olive oil interaperitoneally once a day for one week and the ALLO/ CsA group received 300 µL CsA (1mg/kg/day) interaperitoneally once a day for one week.

Results: Behavioral, functional, biomechanical and gastrocnemius muscle mass showed earlier regeneration of axons in ALLO/ CsA than in ALLO group ($p=0.001$). Histomorphometric and immunohistochemical studies also showed earlier regeneration of axons in ALLO/ CsA than in ALLO group ($p=0.034$).

Conclusion: Administration of CsA could accelerate functional recovery after nerve allografting in sciatic nerve. It may have clinical implications for the surgical management of patients after nerve transection in emergency conditions.

Keywords: Peripheral nerve repair; Sciatic; Cyclosporine A; Allograft.

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Introduction

Peripheral nerve regeneration where there is considerable nerve tissue loss is still a concern

in regenerative medicine [1]. A gap in the peripheral nerve will not permit effective regeneration unless a grafting conduit is used to bridge the defect [2]. Current gold standard for the clinical treatment of

severe peripheral nerve damage involves using an autologous nerve to bridge the defect in injured nerve [3]. This method has been shown to be effective, but has the several disadvantages, including an extra incision for removal of a healthy sensory nerve ultimately resulting in a sensory deficit at the donor site [4, 5].

Surgical therapy in patients with peripheral nerve injuries has not presented changes over the last decades, especially due to the use of autologous grafts, to the development of intraoperative magnification, and to the proven deleterious effects of tension at neural repair site [6]. Despite all the advancements achieved, functional repair results are still imperfect. In addition, the collection of donor nerves produces a new neurological sequel. In extensive defects or in several nerve defects on a same patient, there may not be enough autologous donor nerve to fill that neural failure. With the increasing understanding capacity and with the manipulation of the immune system, non-autologous grafts have been proposed as an alternative method in peripheral nerve reconstructions [6].

The development of therapeutic agents that can promote the rate of nerve regeneration and enhance the degree of functional restitution after injury is important. By accelerating axonal regeneration, the consequences of denervation on target organs (muscle atrophy, loss of sensory receptors, denervation hypersensitivity) would be diminished and functional recovery (following reinnervation) would be more efficient [7].

Immunosuppressants were originally introduced to prevent rejection of allografts. Later, it was discovered that FK506 also possessed nerve regenerative properties when applied systemically and topically loaded at sub-immunosuppressant doses in non-nerve grafts [8-10]. Cyclosporine has been widely employed in organ transplantations in association with other drugs, allowing a significant morbidity reduction when compared to early immunosuppression methods [11]. Mechanism of action of cyclosporine in nerve regeneration remains controversial [12-15]. The local effect of cyclosporine A (CsA) on peripheral nerve regeneration has been investigated after peripheral nerve transection in experimental models immunosuppressed with CsA [16]. Most of these studies were concentrated on local effect of CsA on peripheral nerve regeneration rather than allograft survival [14]. The literature is poor and to the best of our knowledge effect of CsA on allograft survival has not been well investigated to date.

Because of promising beneficial effects of allografting technique and neuroregenerative and immunosuppressive effects of CsA, the objective of the present study was to design a CsA treated allograft in bridging the defects in rat sciatic nerve transection model. Assessment of the nerve regeneration was based on behavioral, functional (Walking Track Analysis), biomechanical, histomorphometrical and

immunochemical (Schwann cell detection by S100 expression) criteria within a 12-week interval after surgery.

Materials and Methods

Experimental Design

Thirty male white Wistar rats weighing approximately 300 g were divided into three experimental groups (n=10), randomly: Normal control group (NC), allograft group (ALLO), CsA treated group (ALLO/CsA). Two weeks before and during the entire experiments, the animals were housed in individual plastic cages with an ambient temperature of $23\pm 3^{\circ}$ 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 CsA

The dose of CsA was determined according to the method described by others [17]. Briefly, the original solution of CsA (Sigam-aldrich, Chemie GmbH, Germany) was 5 mg/mL, so a dilution was performed to reduce the concentration to 1 mg/mL: carrier dilution, 0.2 mL original CsA into 0.8 mL sterile olive oil to give 1 mg/mL.

Grafting Procedure

Animals were anesthetized by intraperitoneal administration of ketamine-xylazine (ketamine 5%, 90mg/kg and xylazine 2%, 5mg/kg). All procedures followed a standard microsurgery technique under magnifying lenses (BIO-ART EQUIPMENTS ODONTOLOGICOS LTDA, Sao Carlos/SP-Brasil). The procedures were carried out based on the guidelines of the Ethics Committee of the International Association for the Study of pain [18]. The University Research Council approved all experiments.

Following surgical preparation in the normal control group (NC) the left sciatic nerve was exposed through a gluteal muscle incision and after careful homeostasis the muscle was sutured with resorbable 4/0 sutures, and the skin with 3/0 nylon. The NC rats received 300 μ L sterile olive oil interaperitoneally once a day for one week. In the ALLO group the left sciatic nerve was exposed through a gluteal muscle incision and transected proximal to the tibio-peroneal bifurcation where a 10 mm segment was excised. The same procedure was performed in the ALLO/CsA group. The harvested nerves of the rats of ALLO group were served as allograft for ALLO/CsA group and vice versa. The proximal and distal stumps of transected sciatic nerve was sutured to the ends of the harvested allograft using 10/0 nylon. The ALLO group animals received 300 μ L olive oil interaperitoneally once a day for one week and the ALLO/CsA group animals received 300 μ L CsA (1mg/kg/day) the same way.

The animals were anesthetized (see above)

and euthanized with transcardial perfusion of a fixative containing 2% paraformaldehyde and 1% glutaraldehyde buffer (pH=7.4) 16 weeks after surgery based on a method described by Kasukurthi *et al.*, [19] and gastrocnemius muscle mass measurement, biomechanical testing, histomorphometric and immunohistochemical assessments were performed.

Behavioral Testing

Functional recovery of the nerve was assessed using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale for rat hind limb motor function [20]. Although BBB is widely used to assess functional recovery in spinal cord injured animals, however, it has been demonstrated that it could be most useful in assessment of never repair processes in peripheral nerve injuries [21]. Scores of 0 and 21 were given when there were no spontaneous movement and normal movement, respectively. A score of 14 shows full weight support and complete limbs coordination. BBB recordings were performed by a trained observer who was blinded to the experimental design. The testing was performed in a serene environment. The animals were observed and assessed within a course of a 4-minute exposure to an open area of a mental circular enclosure. BBB scores were recorded once before surgery in order to establish a baseline control and again weekly thereafter to assess functional recovery during 12 weeks.

Functional Assessment of Reinnervation Sciatic Functional Index (SFI)

Walking track analysis was performed 4, 8 and 12 weeks after surgery based on the method of others [22]. The lengths of 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) of each animal was calculated by the following formula:

$$SFI = -38.3 \times (EPL - NPL) / NPL + 109.5 \times (ETS - NTS) / NTS + 13.3 \times (EIT - NIT) / NIT - 8.8$$

In general, SFI oscillates around 0 for normal nerve function, whereas around -100 SFI represents total dysfunction. SFI was assessed in the NC group and the normal level was considered as 0. SFI was a negative value and a higher SFI meant the better function of the sciatic nerve.

Static Sciatic Index (SSI)

SSI is a time-saving digitized static footprint analysis described by others [21] good correlation between the traditional SFI and the newly developed static sciatic index (SSI) and static toe spread factor (TSF), respectively, has been reported by others [21]. The SSI is a time-saving and easy technique for accurate functional assessment of peripheral nerve regeneration in rats and is calculated using the static factors, not considering the print length factor (PL), according to the equation:

$$SSI = [(108.44 \times TSF) + (31.85 \times ITSF)] - 5.49$$

Where:

$$TSF = (ETS - NTS) / NTS$$

$$ITSF = (EIT - NIT) / NIT$$

Like SFI, an index score of 0 was considered normal and an index of -100 indicated total impairment.

Biomechanical Testing

The nerve segment from NC animals and the allografts of ALLO and ALLO/CsA were harvested and placed in a normal saline bath at room temperature. The samples were then fixed between frozen fixtures in a mechanical apparatus. The TA.XTPlus Texture Analyzer mechanical test device was used for the assessment (Stable Micro Systems, Surrey GU7 1YL, UK). After 5 minutes, the frozen fixtures were tightened to ensure that no slippage occurred during testing. The initial length was set to 10 mm. Each sample was stretched at a constant rate of 1 mm/min. The load and displacement were sampled 5 times per second. Each sample was stretched to complete tensile failure. Samples were kept wet moist during testing using a drop of normal saline solution to the nerve segments.

Measurement of Gastrocnemius Muscles Mass

Recovery assessment was also indexed using the weight ratio of the gastrocnemius muscles 16 weeks after surgery. Immediately after sacrificing of animals, gastrocnemius muscles were dissected and harvested carefully from intact and injured sides and weighed while still wet, using an electronic balance. Two independent observers unaware of the analyzed group made all measurements.

Histological Preparation and Quantitative Morphometric Studies

The harvested segments were fixed in 2.5 percent glutaraldehyde. The grafts were then embedded in paraplast paraffin, cut in 5 μ m and were next stained with toluidine blue. Morphometric analysis was carried out using an image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA). Equal opportunity, systematic random sampling and two-dimensional dissector rules were followed in order to cope with sampling-related, fiber-location-related and fiber-size related biases [23].

Immunohistochemical Analysis

In this study, anti-S-100 (1:200, DAKO, USA) was used as marker for myelin sheath. Specimens were post fixed with 4% paraformaldehyde for 2h and embedded in paraffin. Prior to immunohistochemistry 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 non-specific immunoreactions the sections were incubated with normal swine serum (1:50, DAKO, USA). Sections were then incubated in

S-100 protein antibody solution for 1h at room temperature. They were washed three times with PBS and incubated in biotinylated anti-mouse rabbit IgG solution for 1h. Horseradish peroxidase-labelled secondary antibody was applied for 1 h. After that all sections were incubated with 3,3'- diaminobenzidine tetrahydrochloride chromogene substrate solution (DAB, DAKO, USA) for 10 min. The results of immunohistochemistry were examined under a light microscope.

Statistical Analysis

Experimental results were expressed as means±SD. Statistical analyses were performed using PASW 18.0 (SPSS Inc., Chicago, IL, USA). Model assumptions were evaluated by examining the residual plot. Results were analyzed using repeated measures and a factorial ANOVA with two between-subjects factors and. Bonferroni test for pairwise comparisons was used to examine the effect of time and treatments. The differences were considered significant when $P < 0.05$.

Results

BBB Recovery

In order to assess hind limb recovery the open field

locomotor was used. Figure 1 shows BBB scores compared to the baseline. All experimental groups, except for sham, showed the greatest degree of functional deficit one week after surgery. The CsA-treated group showed significant improvement in locomotion of the operated limb compared to the control group during the study period ($p=0.001$).

Recovery of Sciatic Nerve Function and Reinnervation SFI Outcome

Figure 2 shows sciatic function index (SFI) values in experimental groups. Prior to surgery, SFI values in both groups were near zero. After the nerve transection, the mean SFI decreased to -100 due to the complete loss of sciatic nerve function in all animals. At the end of the study period the statistical analyses revealed that the recovery of nerve function was significantly ($p=0.002$) different between ALLO and ALLO/CsA groups and administration of CsA improved functional recovery in the course of time.

SSI Outcome

Changes in SSI were similar to those observed in SFI, indicating significant deficit following the sciatic nerve transection (Figure 3). Changes in SSI were significant within the weeks of recovery ($p=0.002$).

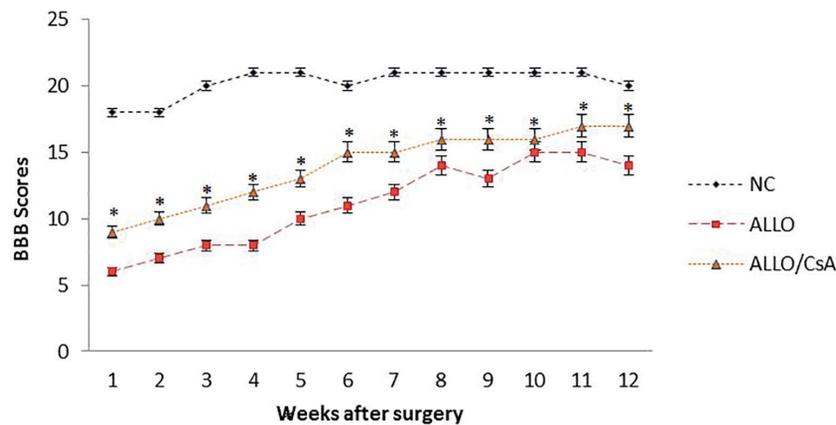


Fig. 1. BBB score for all experimental groups. Administration of CsA with allografting gave better scores than in ALLO group. Standard error at each data point is shown with bars. * $p=0.001$ vs ALLO group.

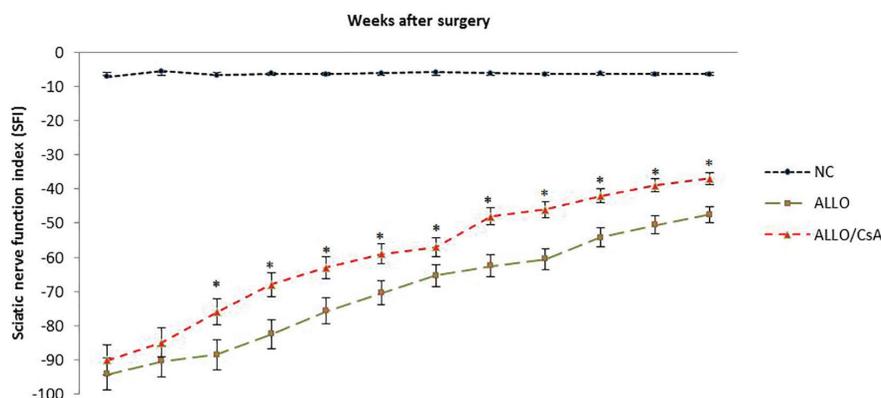


Fig. 2. Diagrammatic representation of effects on the sciatic nerve function index (SFI) in each experimental group during the study period. Statistically significant improvement ($p=0.002$) was observed in functional recovery of the sciatic nerve in CsA treated animals at the end of the study period. * $p=0.002$ vs ALLO group.

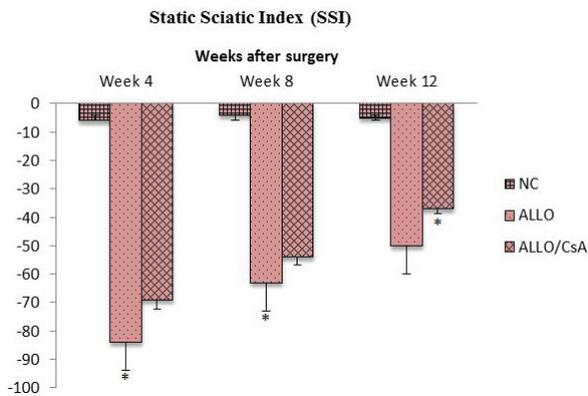


Fig. 3. Bar graph indicating static sciatic index (SSI) values in each experimental group during the study period. Administration of CsA gave better results in functional recovery of the sciatic nerve compared to ALLO group. Data are presented as mean±SD. * $p=0.002$ vs ALLO group.

The contrasts indicate SSI values in group ALLO/CsA at week 12 to differ significantly from those obtained from ALLO, a trend also noticed for SFI ($p=0.002$).

Biomechanical Measurements

Biomechanical analyses of regenerated nerves for each of the experimental groups are shown in Table 1. Maximum pull force (F_{max}) of normal sciatic nerve was found to be 5.50 ± 0.40 . F_{max} in ALLO/CsA group was not significantly higher than that in ALLO group ($p > 0.05$). Tensile strength, the amount of force per unit of initial cross-sectional area at tensile failure, was measured based on F_{max} and nerve cross sectional area. Sixteen week assessment revealed tensile strength in ALLO/CsA was not higher than those in ALLO group ($p = 0.001$). No significant difference was observed in ultimate strain, the amount of elongation divided by the initial specimen length achieved at the point of tensile failure, between ALLO/CsA and ALLO groups ($p = 0.001$). Toughness, reflecting the properties of anti-deformation and anti-fracture of nerve, was determined by the nerve itself and could reflect “looseness” or “toughness” of nerve.

Table 1. Biomechanical analyses of sciatic nerve in each of the experimental groups: Values are given as mean±SD.

Groups	Maximum Pull Force (N)	Tensile Strength (MPa)	Ultimate Strain	Toughness (N/mm)
NC	5.53 ± 0.50	7.32 ± 1.10	0.58 ± 0.05	1.23 ± 0.30
ALLO	3.28 ± 0.36	3.55 ± 0.17	0.27 ± 0.04	0.47 ± 0.22
ALLO/CsA	4.70 ± 0.27	4.77 ± 0.14	0.49 ± 0.05	0.78 ± 0.18

*The mean difference is significant at the .05 level vs. ALLO group.

Table 2. Morphometric analyses of sciatic nerve in each of the experimental groups: values are given as mean±SD.

Groups	Axon counts fb/mm ²	Axon diameter (µm)	Myelin sheath thickness(µm)
NC	29500 ± 2105	11.38 ± 0.22	2.50 ± 0.05
ALLO	21403 ± 2306	3.82 ± 0.11	1.05 ± 0.04
ALLO/FK506	24504 ± 2205^a	6.32 ± 0.15^a	1.28 ± 0.05^a

^aThe mean difference is significant at the .05 level vs. ALLO group.

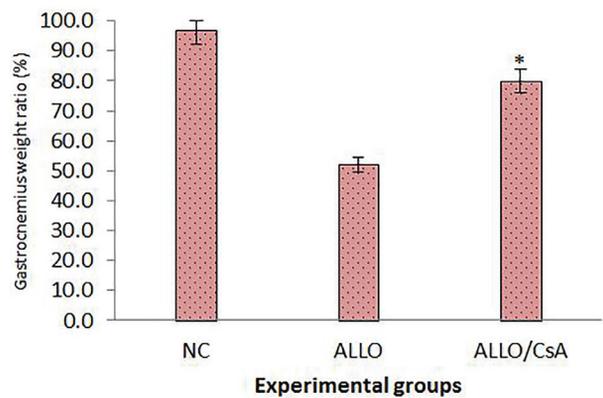


Fig. 4. Measurement of gastrocnemius muscle mass. The gastrocnemius muscles of both sides (operated left and unoperated right) are excised and weighed in the experimental groups at 12 weeks after surgery. * $p=0.001$ vs ALLO group. Data are presented as mean±SD.

Toughness in ALLO/CsA and ALLO group was not significantly different ($p > 0.05$).

Gastrocnemius Muscles Mass Measurement

The mean ratios of gastrocnemius muscles weight were measured. There was statistically significant difference between the muscle weight ratios of ALLO/CsA and ALLO groups ($p=0.001$). The results showed that in ALLO/CsA group muscle weight ratio was bigger than ALLO group and the gastrocnemius muscle weight loss was improved by administration of CsA (Figure 4).

Histological and Morphometric Findings

The Table 2 shows the quantitative morphometric analyses of regenerated nerves for each of the experimental groups. The CsA treated group presented significantly greater nerve fiber, axon diameter, and myelin sheath thickness compared to ALLO animals ($p=0.034$) (Figure 5).

Immunohistochemistry

Immunoreactivity to S-100 protein was extensively observed in the cross sections of regenerated nerve

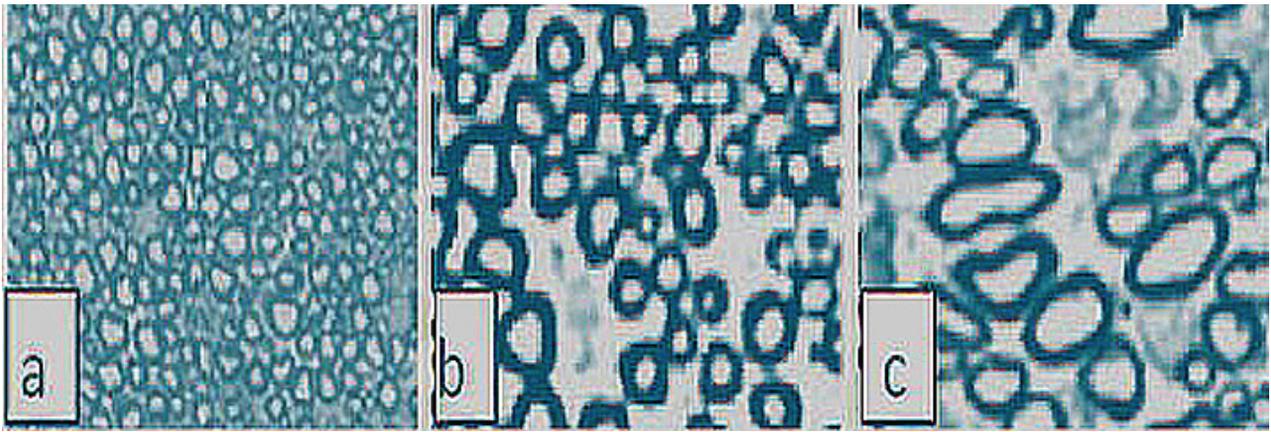


Fig. 5. The light micrograph of representative cross section taken from (a) midpoint of normal sciatic nerve (NC), (b) (ALLO) and (c) (ALLO/CsA) 12 weeks after surgery. (Toluidine blue, $\times 400$)

segments. The expression of S-100 protein signal was located mainly in the myelin sheath. The axon also showed a weak expression indicating that Schwann cell-like phenotype existed around the myelinated axons (Figure 6). In both ALLO/CsA and ALLO groups, the expression of S-100 and the findings resembled those of the histological evaluations.

Discussion

The results of the present study showed that administration of CsA resulted in faster functional recovery of the sciatic nerve during the study period. The assessment and interpretation of the results achieved with nerve allografts are still controversial due to the uncertain histocompatibility between donor and receptor of different grafting technique and of the complexity of quantitative methods for neural regeneration assessment [6]. Others proposed an experimental model to study neural regeneration with allografts in rats, applying a computer-based method for assessing results [24]. Although both morphological and functional data have been used to assess neural regeneration after induced crush injuries, the correlation between these two types of assessment is usually poor [25-27]. Classical and newly developed methods of assessing nerve

recovery, including histomorphometry, retrograde transport of horseradish peroxidase and retrograde fluorescent labeling do not necessarily predict the reestablishment of motor and sensory functions [22, 26, 28, 29]. Although such techniques are useful in studying the nerve regeneration process, they generally fail in assessing functional recovery [26]. Therefore, research on peripheral nerve injury needs to focus on functional assessment. It has been suggested that arrival of sprouts from the proximal stump at the distal nerve stump does not necessarily imply recovery of nerve function. Information taken from BBB scale may be invaluable in evaluation of peripheral nerve process [29]. Results of the present study showed that the CsA treated animals had been improved in locomotion of the operated limb compared to the ALLO group during the study period. Walking track analysis has frequently been used to reliably determine functional recovery following nerve repair in rat models [22]. Left gastrocnemius muscle weight was significantly greater in the ALLO/CsA group than in the ALLO group, indicating indirect evidence of successful end organ reinnervation in the CsA treated animals. In the histological studies, quantitative morphometrical indices of regenerated nerve fibers showed significant difference between ALLO and

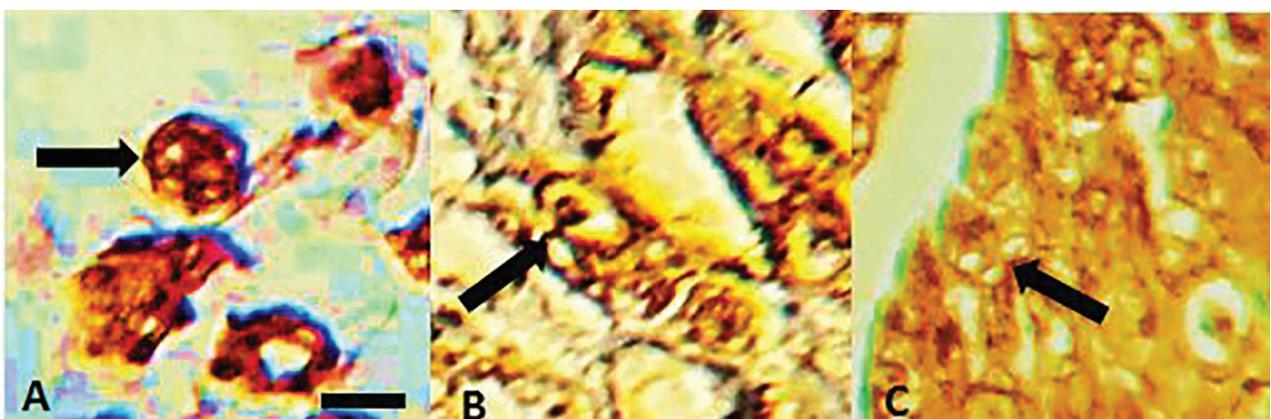


Fig. 6. Immunohistochemical analysis of the regenerated nerves 12 weeks after surgery from middle cable (A) NC, (B) ALLO and (C) ALLO/CsA. There is clearly more positive staining of the myelin sheath-associated protein S- 100 (arrow) within the periphery of nerve, indicating well organized structural nerve reconstruction CsA treated nerve. Scale bar: 10 μm

ALLO/CsA groups indicating beneficial effect of topical CsA on the nerve regeneration. The thickness of myelin showed no significant differences between ALLO/CsA and that of sham operated group from week 8 to the end of the study period. Others in their eight-week period work reported that the regenerated myelinated fibers showed a more mature morphometric profile at 8 weeks [29].

In immunohistochemistry the expression of axon and myelin sheath special proteins was evident in both groups which indicated the normal histological structure. The location of reactions to S-100 in ALLO/CsA group was clearly more positive than ALLO group further implying that both regenerated axon and Schwann cell-like cells existed and were accompanied by the process of myelination and the structural recovery of regenerated nerve fibers.

The ability of CsA to increase nerve regeneration in vivo is reported to be dose-related the rat in systemic administration.¹³ Results of the ability of CsA in peripheral nerve regeneration are different and sometime conflicting in the literature [6, 13, 29-31]. 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. Others showed that the use of cyclosporine was associated with a significant increase of fiber density and percentage of neural tissue on the distal segment of reconstructed nerves in immunosuppressed animals [6, 32].

It has been reported that low dose administration of CsA, 5 mg/kg per day subcutaneously, increased quantitative values of fiber density [6]. 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 distal stump of transected nerve start proliferating and help inflammatory infiltrating cells to eliminate debris and upregulate synthesis of trophic and tropic factors throughout wallerian degeneration, and regeneration is failed or delayed in situations where process of wallerian degeneration is diminished [33]. Host immunosuppression using cyclosporine and predegeneration of nerve grafts were useful techniques for successful installment

and regeneration across nerve allografts in rabbits [30]. CsA accelerates axonal regrowth with systemic administration. Entubulation neuroorrhaphy using CsA loaded chitosan conduit as an in situ delivery system of CsA in bridging the defects has been reported with promising results [16].

Even though our preliminary study shows the neuroprotective action of local CsA in peripheral nerve injuries, determining the molecular mechanisms leading to the neuroprotective action remains needs to be investigated. We have not given the histological and molecular evidence for neuroprotective action of CsA. This may be considered as a limitation to our study. Therefore, the authors stress that the aim of the current investigation was to evaluate clinical treatment potential of CsA on allograft nerve regeneration including functional and biomechanical assessments of the nerve repair, a case not considered in previous studies. The results of the present study indicated that administration of CsA could be of benefit after sciatic nerve allografting. Detailed mechanism of neuroprotective action remains to be investigated.

The experimental model presented here is reproducible for the study of nerve allografting and the behavioral and functional methods could be effectively used for the study of peripheral nerve regeneration in allograft models.

The present study demonstrated that administration of CsA could accelerate functional recovery after nerve allografting in sciatic nerve and may have clinical implications for the surgical management of patients after nerve transection in emergency conditions. Thus, dose-response studies should be conducted for CsA to determine the combination of the allograft and the compound that achieve maximal efficacy in nerve allograft models.

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Conflicts of Interest: None declared.

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