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Locally Applied FK506 Improves Functional Recovery in Rats after Sciatic Nerve Transection

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Abstract:

Peripheral nerve injury (PNI) is a serious health concern for society and often causes long lasting disability. Nerve tubulization is recommended when there is too much tension for end-to-end coaptation and the gap between stumps is shorter than 3 cm. The immunosuppressive drug FK506 (Tacrolimus) has demonstrated neuroprotective and neurotrophic actions in experimental models by increasing neurite elongation and accelerating the rate of nerve regeneration in vitro and in vivo. The aim of this study was to evaluate functional recovery through walking track analysis after nerve transection with loss of substance. FK506 was used as a neurotrophic factor to stimulate neuronal growth through the tubulization technique along with a controlled drug delivery system, the alzet osmotic minipumps. Rats (n=36) were randomly assigned to three groups. Group I served as untreated controls. Groups II and III received 0.1 mg/kg/d of FK506 systemically and locally, respectively. After 90 days postoperative results showed the Group III achieved the best functional recovery (highest Sciatic Functional Index – SFI) in comparison with Groups I and II.

Keywords: ***peripheral nerve injury, tubulization, FK506, osmotic pumps.***

1. Introduction

Peripheral nerve injury (PNI) is a serious health concern for society and has posed an intimidating challenge to surgeons since Cruikshank first reported successful repair in 1795. It affects 2.8% of trauma patients, many of whom acquire life-long disability[1]-[3]. Loss of sensory and motor function, accompanied by pain and discomfort not only have functional consequences but also have major social and psychological impact[4]. Peripheral nerve transection results in Wallerian degeneration in all of the axons distal to the injury site, as evidenced by the disintegration of axoplasmic microtubules and neurofilaments. Most of the axons along the distal stumps of transected nerves are reduced to granular and amorphous debris within 24 hours; by 48 hours, the myelin sheath has begun to be transformed into short segments that then form into ovoids. Activated macrophages migrate into the degenerating nerve stumps and phagocyte the disintegrating nerve fibers and myelin. Schwann cells proliferate in response to myelin debris and macrophage-derived cytokines and form longitudinal Schwann cell bands (bands of Bungner) as they divide and remain within the basal-lamina-lined endoneurial tubes[3].

The surgical management of injured peripheral nerves where a gap is present still remains a formidable challenge in reconstructive surgery. Bridging the nerve gap by the interposition of an autogenous nerve graft is the current treatment of choice for such situation. However, the limited availability of donor sites for nerve grafts (sural nerve, approximately 25-30cm), the potentially inappropriate diameter of around 1.5mm of most selected nerves, and their inherent associated morbidity or neuroma formation continue to stimulate researches toward finding a suitable alternative to bridge a gap and to enhance the process of peripheral nerve regeneration[5]. The tubulization technique attempts to create mechanical guidance for the growth of the nerve trunk, and consists in inserting and suturing proximal

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and distal nerve endings into a conduit with an overlap between the stumps[6],[7].

FK506 is one of many neurotrophic factors available. FK506 is an immunophilin ligand and was initially FDA-approved as an immunosuppressive agent for solid organ transplantation in humans. However, it has recently demonstrated neurotrophic effects, a high potential for nerve regeneration and neuroprotection[8]-[11] and fewer side effects than other immunosuppressants[12]-[15] which appear to be independent of its immunosuppressive activity[16].

In vitro, FK506 significantly increases neurite outgrowth from SH-SY5Y neuroblastoma cells and from chick sensory ganglia. Importantly, FK506 enhances nerve regeneration following rat sciatic nerve injury when administrated systemically. Moreover, sustained systemic administration of FK506 in combination with graft therapy or tube repair increases the rate of nerve regeneration and promotes functional recovery[16]. Because of properties, FK506 is now being used to improve peripheral nerve regeneration across nerve allografts[17],[18]. Tacrolimus has powerful effects on the promotion of neural regeneration, and the underlying mechanism is mainly associated with immune inhibition and neurotrophic activity[19].

Functional recovery following experimental nerve injury is notoriously difficult to quantify. The current gold standard in the rat sciatic nerve model and most commonly used method for measuring functional recovery following rat sciatic nerve injury is walking track analysis. This method involves analysis of footprints of the recovering animal, and computation of the sciatic function index (SFI)[20] and was first described by de Medinaceli et al[21] and subsequently modified by Bain et al[22]. The approach they described is utilized increasingly by neuroscientists. It involves the measurement of three relationships: 1) (print length (PL): distance from the heel to the third toe; 2) toe spread (TS): distance from the first to the fifth toe; 3) intermediate toe spread (ITS): distance from the second to the fourth toe, between toes and feet of the hind limb of recovering animals. Different techniques are used to obtain footprints for walking track analysis, ranging from visible footprints in a walkway to digitally recorded footprints[23]-[28].

Herein we measure functional recovery through the walking track analysis after nerve transection with loss of substance using FK506 as a neurotrophic factor.

2. Materials and Methods

2.1 Animals and surgical procedures

Male Wistar rats, weighing 250-300g and 3 months of age were used. These studies were approved by the local animal ethics committee at Pontifical Catholic University of Rio Grande do Sul (PUCRS), Brazil. All animals were kept in plastic cages in a 12 h light and 12 h dark circle. At surgery, the rats were anesthetized with an intraperitoneal injection of Ketamine (75 mg/kg) and Xylazine (10 mg/kg). All surgical procedures were performed aseptically using standard microsurgical techniques and an operating microscope.

Operation of sciatic nerve transection (SNT) was carried out in 36 rats on the right leg by opening the skin over a length of 2 cm in the proximal half of the line between trochanter major and knee joint. The m. vastus lateralis and m. biceps femoris were separated by blunt preparation techniques and the sciatic nerve could now be seen from where it emerges from under the m. gluteus maximus and runs over the m. semimembranous and m. semitendinous[29]. All animals underwent left sciatic nerve exposure, transection (10mm gap) and repair with silicon tubulization. The exposed distal and proximal nerve stumps were pulled into the silicon tubule (15mm) and secured with one 9-0 nylon suture (Ethicon, Somerville, NJ). The alzet osmotic minipumps were placed subcutaneously in the back of each rat. The experimental set-up is described in Table 1.

Table 1: Experimental set-up

Group	Description	Number of rats
I	Untreated controls	12
II	FK506 via Alzet minipump (systemic)	12
III	FK506 via Alzet minipump (local)	12

2.2 Drug Administration

The rats of groups II and III received therapy with FK506 (FK-506 Tacrolimus monohydrate, Sigma-Aldrich); Tacrolimus was diluted to a concentration of 10 mg/ml in anhydrous alcohol. Rats of group II received a 0,1 mg/kg/day FK506 solution via Alzet minipump (model 2006, Alzet, Cupertino, CA) systemically whereas rats of group III received 0,1 mg/kg/day FK506 solution via Alzet minipump locally (a catheter was adapted to deliver the solution locally, inside the silicon tubule). The delivery rate of the FK506 solution via Alzet osmotic pumps was 0.15µl/h during 6 weeks. After this period, the pumps were replaced to reach the time of the experiment (the treatment started at the day of the surgery and lasts 12 weeks).

2.3 Evaluation

2.3.1 Functional Assessment

A standardized walking track analysis (WTA) technique allowed for assessment of hind limb recovery by examination of footprint patterns. Animals were tested in a confined walkway with a dark shelter at the end of the corridor by an observer unaware of group assignment. After three conditioning trials during which rats often stopped to

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explore the corridor they walked steadily to the dark shelter. The bottom of the track was lined with strips of paper. After the hind feet of the animals were painted with a black paint *(Tempera Guache, Acrilex)*, they were allowed to ambulate down the 43 x 8,7 x 5,5 cm wooden corridor. The paw prints appeared immediately on the paper. Three to four footprints from both sides appeared on each track. Sciatic nerve function was evaluated on days 30, 60 and 90 after surgery. For the calculation of sciatic functional index (SFI) the following footprint parameters were measured: the distance from one foot to the opposite foot (TOF); the footprint length (PL); the distance from the first to fifth toes (the toe spread or TS); the distance from the second to fourth toe (the intermediary spreading or IS). Data was collected for both the normal (N) and the experimental (E) feet. The sciatic functional index was calculated by using the following formula[30]:

$$SFI = -38.8\left(\frac{EPL - NPL}{NPL}\right) + 109.5\left(\frac{ETS - NTS}{NTS}\right) + 13.3\left(\frac{EIT - NIT}{NIT}\right) - 8.8$$

The SFI is expressed in units of functional deficit. Thus, normal SFI was defined as 0–10 and 100 represented complete loss of nerve function.

2.3.2 Histomorphometric analysis

At the end of the study, animals were reanesthetized, the operated nerve was reexposed, and excised *en bloc* to include proximal and distal host segments. Immediately following nerve harvest, animals were sacrificed with a lethal intravenous dose of Pentobarbital (Delmarva Laboratories, Midlothian, VA). The sample was then fixed in glutaraldehyde (2,5%) in 0.1 M phosphate buffered saline (pH 7.4, 48h, 25°C), postfixed in OsO4 (1%, 2h, 4°C), and dehydrated through ethanol series. After the first dehydration, the tissues were stained with propylene oxide (1,2-epoxypropane) as a transition solvent to facilitate resin infiltration. The samples were then processed for embedding in Epon. Transverse semithin sections (0.5 µm) of the entire nerve at midpoint, proximal and distal to the guide were made with an ultramicrotome (LKB 6802), stained with toluidine blue and examined under light microscopy. The investigators remained blinded to the treatment groups during examination of the slides with a Zeiss primostar microscope equipped with a AmScope MT500 of 5mpx camera to computer and utilizing the AmScope MT Software 3.0.0.6 Integrated Image Analysis Systems Morphometry program. This program was used to calculate the proximal, medial and distal nerve segment areas and perimeters (mm^2).

2.3.4 Statistical Analysis

All values are expressed as mean ± SD. A one-way analysis of variance (ANOVA) followed by Student Newmaln-Keuls test (a 5% significant value was accepted at a p-value of <0.05), Mauchly sphericity test, Between-Subjects test, Within-Subjects Effects test, Multivariate test and Bonferroni test was used to compare the data measured by comparison of the normal and the experimental hind limb of the rats using SPSS software version 11.0.

3. Results

Operations were performed on 36 animals, and 34 were able to be evaluated at the 30th, 60th and 90th postoperative day. In the I and III groups, one animal could not be evaluated for walking track analysis after automutilating two toes on the experimental foot on the 60th postoperative day making analysis of walking tracks impossible. At necropsy, mild to moderate fibrosis was observed covering the silicon tubule implantation site. All of the silicon tubules were intact. Functionally, group III demonstrated better rates of recovery (Table 2 and Figure 1).

Table 2: Summary of functional recovery based on Sciatic Functional Index (SFI).

	Group	n	Mean ± SD	p-value
SFI 30 days	Untreated Control	11	-74.029 ± 6.377	0.009**
	Systemic	12	-83.002 ± 5.651	
	Local	11	-77.690 ± 7.482	
	Total	34	-78.380 ± 7.360	
	Untreated Control	11	-69.262 ± 6.886	0.212
	Systemic	12	-70.411 ± 6.363	
	Local	11	-65.394 ± 7.493	
SFI 60 days	Total	34	-68.416 ± 7.044	
	Untreated Control	11	-66.410 ± 8.214	0.000**
	Systemic	12	-50.784 ± 3.799	
	Local	11	-47.328 ± 2.264	
SFI 90 days	Total	34	-54.721 ± 9.809	

** *statistical significance (P < 0.05).*

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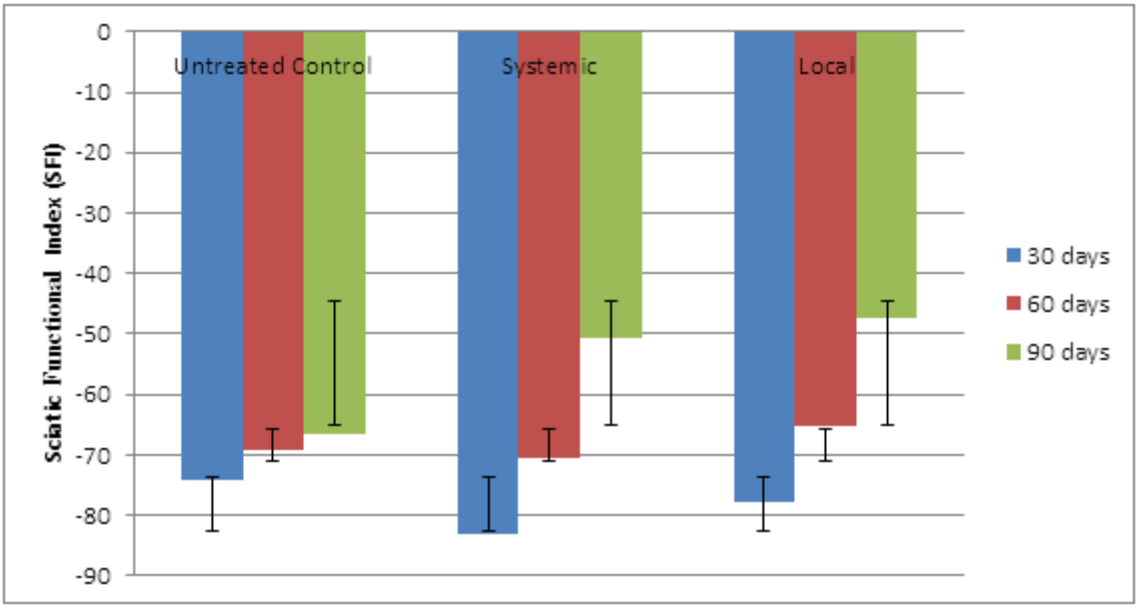

Figure 1: Bar graph depicting the mean functional recovery in terms of SFI of each group after sciatic nerve transection and repair.

In addition, time of evaluation was also important showing better SFI results in 90 days (Figure 2)

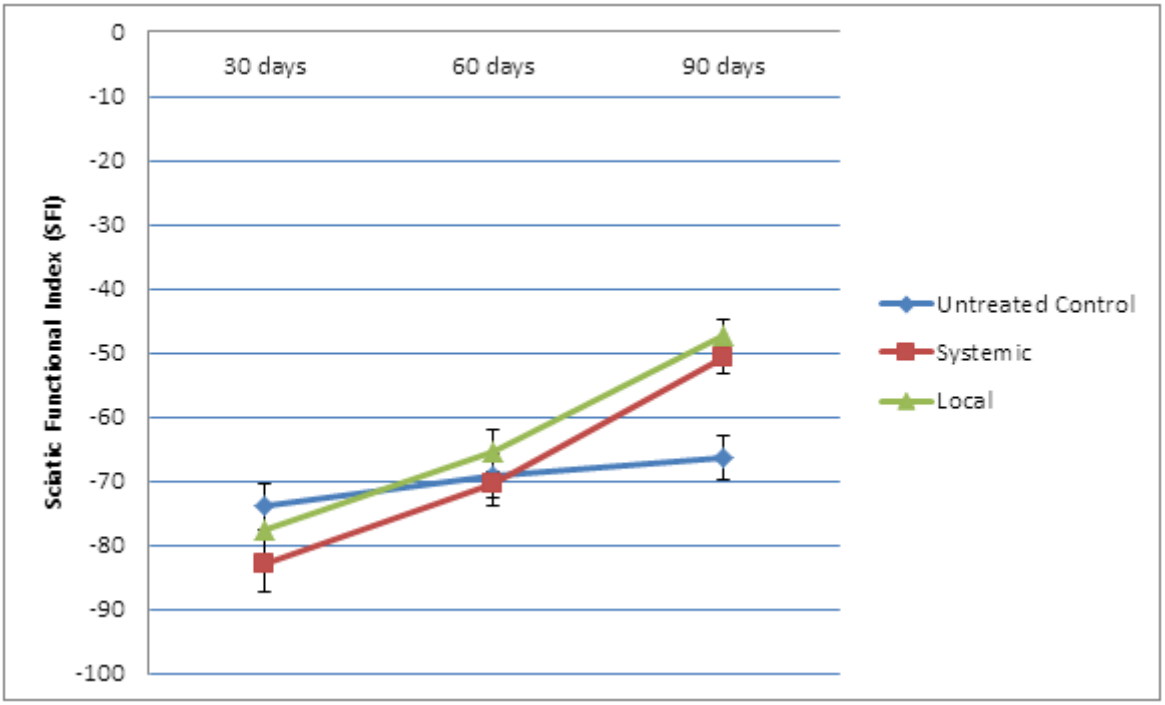

Figure 2: Sciatic Functional Index (SFI) on period of evaluation.

Histomorphometric analysis revealed that mean nerve perimeter and area were larger for mice in Group II than for mice in the other groups. No statistically significant differences were seen comparing groups I and III (Table 3).

Table 3: Summary of morphometric measurements (perimeter and area) according to the group.

Group	Perimeter (µm) Mean ± SD	Area (μm^2) Mean ± SD
Untreated Control	1750.448 ± 312.042	296986.258 ± 136000.247
Systemic	2856.798 ± 254.781	678604.297 ± 136000.247
Local	1721.401 ± 235.882	393344.156 ± 101368.599

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Considering the segment of the nerve, the medial had the smallest mean both for the perimeter and area when compared with all other segments. No statistically significant differences were seen between the proximal and distal. No statistically significant differences were also seen between the medial and distal, only when comparing the segments proximal and medial (Table 4 and Figure 3).

Table 4: Summary of morphometric measurements (perimeter and area) according to the segment of the nerve.

Segment	Perimeter (µm) Mean ± SD	Area (µm^2) Mean ± SD
Proximal	2528.016 ± 226.651	649232.239 ± 124616.167
Medial	1466.065 ± 180.560	195477.331 ± 47992.172
Distal	2334.565 ± 320.325	524225.140 ± 127979.496

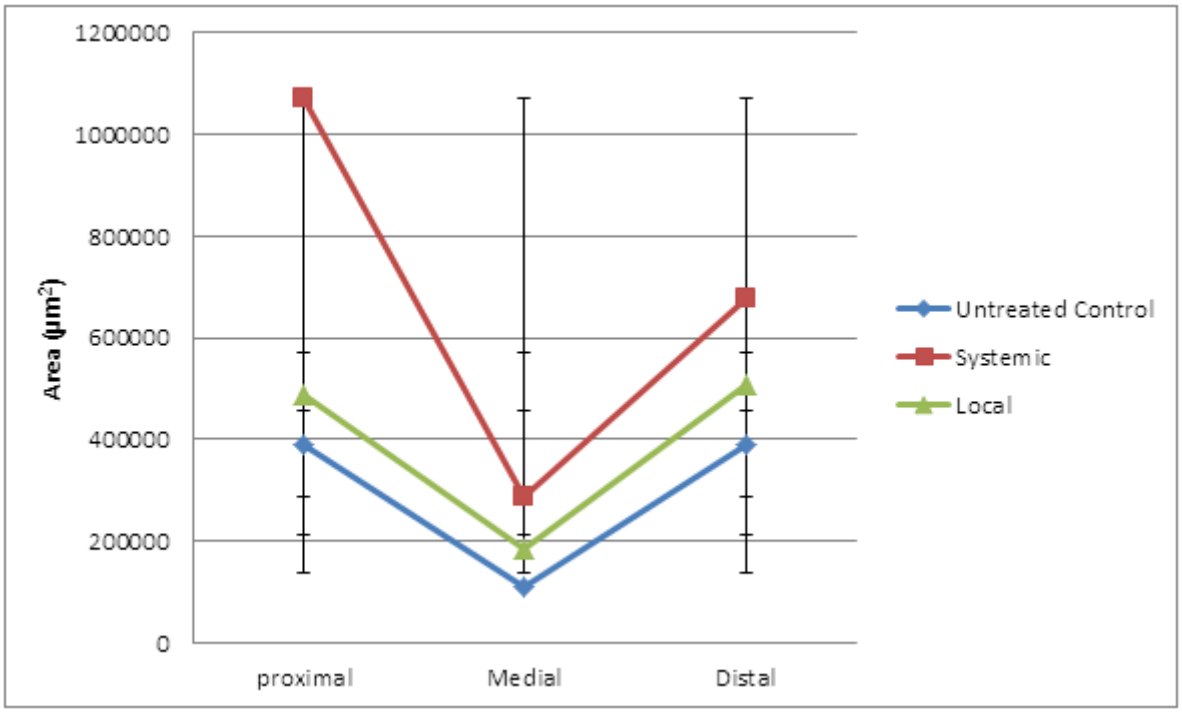

Figure 3: Mean area (µm2) according to experimental groups and the segment of the nerve.

4. Discussion

The main aims of this study were, (1) to evaluate the use of osmotic pumps for delivering neurotrophic drugs in a constant rate, (2) to evaluate the use of FK506 at low-dose along with the tubulization technique as a potential neurotrophic factor for nerve repair, (3) to investigate if the route of administration (systemic or local) affects drug effectiveness, and (4) the improvement of functional recovery after nerve transection using FK506.

Timing is a critical consideration in the study of nerve regeneration once most of the axons along the distal stumps of transected nerves are reduced to granular and amorphous debris within 24 hours. Accelerating nerve regeneration has the potential to optimize functional recovery through early reinnervation and salvaging of functional motor end-plates. Conversely, impaired nerve regeneration not only delays functional recovery but may adversely impact the degree of recovery that is ultimately achieved. A large body of experimental work investigates approaches to enhance nerve regeneration with the goal of improving outcomes after nerve injury[26].

The experimental model studied used FK506 in a silicon tubule repair of severed sciatic nerves with a narrow (10 mm) gap. The silicon tubule was chosen for our model of nerve repair because it enables a closed system that ensures the transected nerve ends are bathed with a precise concentration of matrix factors. This arrangement follows previous studies which showed histomorphologic, immunocytochemical, and electrophysiologic enhancement when matrix factors are used in conjunction with silicon tubulization[31].

The addition of FK506 at low-dose improved functional recovery of transected nerves. The dose chosen for animals treated with FK506 has been shown to produce a maximal response in a nerve allograft model. Quicker regeneration has been documented in animals treated with much higher

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doses of FK506, but we wished to observe any effects on nerve regeneration at low, potentially nonimmunosuppressive doses in search of clinical applications for nerve injuries that otherwise would not require immunosuppression[32].

Various methods have been used in the past to assess recovery from peripheral nerve injury. These include morphometric, electrophysiological, biochemical, and histological analyses. Despite providing useful information, these tests do not measure the most important criteria, functional recovery. The reason why morphometric outcomes do not correlate with functional recovery may be a result of incomplete nerve regeneration or significant misdirection of the regenerating nerve fibers[33].

The concept of walking track analysis as a method of assessing the function of the rat sciatic nerve has been described in detail elsewhere. Briefly, this gait analysis is based on the fact that rats normally walk on their digits and metatarsal footpads. Print length is therefore short in normal animals. Sciatic nerve lesions cause variable loss of both extensors and flexors of the foot. This deficit will cause the foot to drop to the ground and thus change the footprint. In this way footprints can be used to assess sciatic nerve function. Shortening of the footprint is thus a good sign of nerve recovery.

SFI has been widely used to assess motor nerve function and recovery following surgical repair, transection, crush injury, and intraneural injection. It has been shown to correlate with the severity of nerve injury in rats, with muscle strength; it has been validated as reliable, sensitive and reproducible[33]-[37].

According to functional recovery, SFI results showed an improvement after 90 days postoperative. This means that there is a clear interaction between time of treatment and experimental groups. After 60 days postoperative, all groups showed almost the same SFI value pointing that this time would not be enough to allow the growth factor action and consequent nerve regeneration. It can also be verified that, if the treatment were to be extended to 120 or 150 days, the SFI would most likely be even better. When compared all groups in 30, 60 and 90 days, p-value was significant (1% level) in 30 and 90 days, showing that in 60 days there is equilibrium among the three experimental groups. Groups II and III had better functional recovery whereas group I showed minor variation in the SFI. Rustemeyer and co-workers observed improvement in SFI in 60 and 90 days postoperative through systemic (intramuscular injection) FK506 treatment at the same dose rate used in this study (0,1 mg/kg/d). Due to the lack of other studies which employed FK506 along with tubulization technique and osmotic devices, these data need to be investigated and compared in future studies.

While small animals are vital to research on nerve regeneration; work using these models must be undertaken with an informed perspective on the unique biology of small animals and how it differs from that of higher animals. When rodents are evaluated at a late time point, beneficial treatments may be overlooked as conferring no advantage. A more pervasive problem is the tendency to overestimate the efficacy of a new therapy that appears equivalent to a gold standard at late time points. These observations underscore the importance of rigorous controls and appropriate timing in functional and morphometric assessments of nerve regeneration[26].

In conclusion, we showed that daily FK506 low-dose treatment in rat models improves recovery of function as compared to no treatment. The degree of functional recovery as measured by SFI was better when FK506 was administered locally rather than systemically. To our knowledge, this was the first study investigating local application of FK506 in a constant rate.

5. Conclusion

The systemic via showed the best morphometric results indicating that, when the untreated control group was related to the local group, there were no differences. In other words, the presence of FK506 in the local group comparing these two groups was not significant. On the other hand, functional recovery was achieved in the local group, as evidenced by the SFI.

New studies using FK506 together with the technique of tubulization and continuous dispensing through osmotic minipumps are necessary to prove their efficacy in peripheral nerve lesions with loss of substance.

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