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RESEARCH ARTICLE

Effects of Valproic Acid on Axonal Regeneration and Recovery of Motor Function after Peripheral Nerve Injury in the Rat

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Abstract

Background: Valproic acid (VPA) is used to be an effective anti-epileptic drug and mood stabilizer. It has recently been demonstrated that VPA could promote neurite outgrowth, activate the extracellular signal regulated kinase pathway, and increases bcl-2 and growth cone-associated protein 43 levels in spinal cord. In the present research we demonstrate the effect of VPA on peripheral nerve regeneration and recovery of motor function following sciatic nerve transaction in rats.

Methods: The rats in VPA group and control group were administered with valproic acid (300mg/kg) and sodium chloride respectively after operation. Each animal was observed sciatic nerve index (SFI) at 2-week intervals and studied electrophysiology at 4-week intervals for 12 weeks. Histological and morphometrical analyses were performed 12 weeks after operation. Using the digital image-analysis system, thickness of the myelin sheath was measured, and total numbers of regenerated axons were counted.

Results: There was a significant difference in SFI, electrophysiological index (motor-nerve conduct velocity), and morphometrical results (regenerated axon number and thickness of myelin sheath) in nerve regeneration between the VPA group and controls ($P<0.05$).

Conclusions: The results demonstrated that VPA is able to enhance sciatic nerve regeneration in rats, suggesting the potential clinical application of VPA for the treatment of peripheral nerve injury in humans.

Key words: Bcl-2, Growth cone-associated protein 43, Myelin, Rat, Sciatic nerve index, Valproic acid

Introduction

Valproic acid (VPA) is used to be an effective anti-epileptic drug and mood stabilizer. It has recently been demonstrated that VPA could promote neurite outgrowth, activate the extracellular signal regulated kinase pathway, and increases bcl-2 and growth cone-associated protein 43 (GAP-43) levels in spinal cord. We hypothesized that VPA could enhance axonal regeneration in the rat. In the present research we demonstrate the effect of VPA on peripheral nerve regeneration and recovery of motor function following sciatic nerve transaction in rats. The right sciatic nerve was sharply transected 10 mm distal to the sciatic notch in the rat model and reconnected with four epineurial 9/0 nylon sutures. The rats in VPA group and control group were administered with valproic acid (300mg/kg) and sodium chloride respectively after operation. Each animal was observed sciatic nerve index (SFI) at 2-week intervals and studied electrophysiology at 4-week intervals for 12 weeks. Histological and morphometrical analyses were performed at the end of the experiment, 12 weeks after operation. Using the digital image-analysis system, thickness of the myelin sheath was measured, and total numbers of regenerated axons were counted.There was a significant difference in SFI, electrophysiological index (motor-nerve conduct velocity), and morphometrical results (regenerated axon number and thickness of myelin sheath) in nerve regeneration between the VPA group and controls ($P<0.05$). The results demonstrated that VPA is able to

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enhance sciatic nerve regeneration in rats, suggesting the potential clinical application of VPA for the treatment of peripheral nerve injury in humans.

Recent studies have shown that locally applied neurotrophins can improve survival of damaged neurons and regeneration of axons in the central and peripheral nervous systems in rats (1-3). However, the beneficial effects of neurotrophins are limited by enzymatic degradation and the characteristic of penetrating into the central nervous system hardly through the blood-brain barrier (BBB) as well (4-6). In addition, the neurotrophins have the quite side effect, damaging the liver and kidney when delivered at the required high-dose for a long time. Therefore, it is urgent to find a new material that can pass through the BBB easily and produce neurotrophin-like effects on nerve regeneration with less toxicity problems.

VPA is used to be an effective anti-epileptic drug and mood stabilizer (7-9). Bowen *et al* reported that VPA, at a clinically relevant therapeutic concentration, produces effects similar to those of neurotrophic factors and promotion of neurite growth and neuron survival. VPA possesses the natural characteristic of penetrating rapidly through the BBB because of its small molecular weight (10, 11). Recently, it was founded that VPA exerted neuro-protective effects. Yuan *et al* demonstrated that VPA at therapeutic concentration produces effects similar to neurotrophic factors, namely activation of the ERK pathway and promotion of neurite growth and cell survival (12).

Based on the results in vitro studies, we hypothesized that VPA was able to enhance sciatic nerve regeneration in rats administered VPA systemically. In order to test this hypothesis, in the present study we delivered systemic VPA to the rats following sciatic nerve axtomy and subsequent repair at a clinically relevant dosage. Then, we observed the effect of VPA on axonal regeneration and recovery of motor function in the rat.

Materials and Methods

Animals

A total of 30 male healthy Sprague Dawley rats (SPF grade, license no. 2005-2006), weighing 200-250 g, were provided by Experimental Animal Center at Medical school of Wuhan University.

VPA was provided by the Hengrui Pharmaceutical Factory, Jiangsu. All other chemicals used in the present experiment were of analytical reagent grade or better, and were obtained from the usual commercial sources.

Animal Model Preparation

The animal model preparation was carried out in the Central Laboratory of PuAi Hospital of Wuhan. Rats were anesthetized intraperitoneally with 100g/L sodium Chloral Hydrate (300 mg/kg). All procedures were performed using aseptic techniques and employed standard microsurgical techniques under a 10× operating microscope.

The left sciatic nerve was exposed though a dosal/gluteal splitting incision. Sciatic nerves of rats were transected at 1 cm below infrapiriform foramen and reconnected with four epineurial 9/0 nylon sutures (13-15). Muscular deep fascia was sutured with No.0 suture following haemostasis was performed and skin was closed with No.1 suture.

All rats having received operation were randomized divided into two groups. In VPA group (n=15), the surgical procedure was followed by oral administration of VPA dissolved in drinking water at a dose of 300 mg/kg daily. And, in control group (n=15), rats were administrated by sodium chloride orally.

All rats were fed at 26-28° and humidity of 50%-60% and light-controlled conditions (12:12 h light-dark cycle) in the Experimental Animal Center of Wuhan University. All procedures used were in strict accordance with the National Guidelines for Medical Experimental Animal Management.

Evaluation

Total 30 rats were involved in the experiment, and all of them enter the stage of result analysis, without any loss. Each animal was observed sciatic nerve index (SFI) at 2-week intervals and studied electrophysiology at 2-week intervals for 12 weeks (16). Histological and morphometrical analyses were performed at the end of the experiment, 12 weeks after operation.

Functional Observation

To assess the recovery of motor function, a brightly lit walkway (9.5 cm wide, 42 cm long with 15-cm-high walls) with a dark box at one end was constructed. The rats were allowed to move through the walkway to the bright end several times to be familiar with the apparatus. The hind limbs of experiment rats were dipped in blue ink. Then, the rats were allowed to walk through the walkway, leaving footprints on white paper. Toe spreads and paw length were measured and the SFI was calculated according to the Bain-Mackinnon-Hunter SFI formula:

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

Where PL = print length in mm, TS = spread in mm between 1st and 5th toes, IT = spread in mm between 2nd and 4th toes, and E and N indicate experiment and normal hind foot, respevtively (17).

Electrophysiological Studies

The electrophysiological studies were carried out under anesthesia. A electromyography machine was used here. Stimulus (6 mA at 1 Hz) was delivered under a constant room temperature of 21-25°. One of the bipolar stimulating electrodes was placed at the point proximal to the nerve transection and the other one at the point where the peroneal nerve distributes the peroneal muscle. The distance of the two electrodes was set at 2.0 cm. Recording electrodes were placed in the belly of the tibialis anterior muscle. When the nerve was stimulated by electrodes, motor-nerve conduct velocity could be obtained.

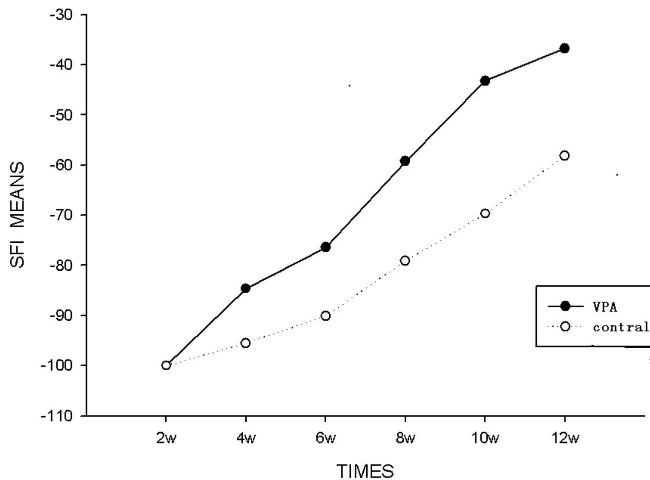

Figure 1. Observation of SFI Means following operation. Reconnected nerves remained nearly completely nonfunctional for first 2 weeks after transection in both groups. From 4--12 weeks, SFI evolved from deep to moderate dysfunction in both groups. However, better recovery of function was observed in VPA group.

Histological and Morphometrical Analysis

Observation of nerve by PTAH staining

All animals were sacrificed for histological and morphometrical analysis following electrophysiological recordings. Nerve specimens (0.5 cm proximal to the tip and 0.5 cm distal to the tip of the sciatic nerve) were prepared for PTAH test. The detailed procedures were as follows: Specimens embedded paraffin were given routine deparaffin into distilled water, dropped into 0.25% permanganate solution for 5 minutes. Then the specimens were washed by distilled water and bleached for 5 minutes in 2.5% oxalic acid. After washed by distilled water several times, specimens were placed in haematoxylin solution combined with phosphotungstic acid for 12~24 h. Following degenerated by 95% alcohol, the specimen were toasted at 60°. AT last, they were made to be transparent and mounted by xylene. The 2-μm-thick serial sections were observed under light microscopy (LM).

Observation of nerve by methylene blue-basic fuchsin staining

The 5-mm length of sciatic nerve 5 mm distal to the repair site and 5 mm proximal to the repair site were respectively removed by section and fixed in 1% glutaraldehyde in 0.1 M phosphate-buffered saline for 2 h, postfixed in 2% osmium tetroxide for another 12 h, dehydrated in a graded ethanol series, and embedded in Epon 812 resin. Each 1-μm-thick section stained with 1% methylene blue was observed under LM. Meanwhile, specimens were cut into 70~90-nm-thick slices on an ultramicrotome for transmission electron microscopy (TEM). Sections were stained with lead citrate and uranyl acetate by the Reynolds method, and then examined by TEM (HITACHI H-600, Japan).

Quantitative analysis was performed on cross sections of the distal nerve specimens. In order to minimize the error, at 400×magnification, 6 measurement windows were randomly selected by an observer blinded to the experiment to counted per nerve, using a digital image-analysis system linked to Image Pro Plus (Media Cybernetics, Silver Spring, MD) morphometry software. Analysis of the digitalized information, based on gray and white scales, was performed by the computer software. Axons were identified by the deeply stained color. A total area of myelinated fibers was measured for each nerve specimen. Thickness of axon sheaths on cross section was also measured by the morphometric computer software.

Table 1. Data of electrophysiological studies ($\bar{x}$ ± s, n=15)

Group	MCV(m/s)		
	4w	8w	12w
VPA group	12.19	20.58	28.42
Control group	9.54	13.85	19.07

* VPA group vs control group, P<0.05

Statistical Analysis

The results were expressed as Mean±SD and data were statistically managed with SPSS 11.5 software. The variables were considered as follows: SFI, MCV, mean regenerated nerve fiber number counted in cross section, and thickness of myelin sheaths. By means of the Kolmogorov-Smirnov test, all variables showed a normal distribution. However, Students t-test on these variables was performed to find statistically significant differences between them. P<0.05 level was set as significant difference.

Results

Functional Observation

Hind limbs prints were analyzed preoperatively. Using the Bain-Mackinnon-Hunter SFI formula, the SFI was obtained at 2-week intervals for 12 weeks postoperative. A score of zero on the index indicated normal function and 100 indicated complete dysfunction (18). In the present study , the SFI of all rats oscillated around -8 before the operation. SFI values began to decrease after the nerve axotomy. However, the SFI figures clearly showed motor function trend to recovery over time a tendency in both groups, but recovery in VPA group appeared better. As the average SFI at 2 weeks was around -100 in both groups, meaning an approximate complete functional loss, but improved to -74.75 (VPA group) and -84.60 (control group) at 4 weeks, and to -55.29 (VPA group) and -73.41(control group; a score compatible with moderate dysfunction of the sciatic nerve) at 12 weeks. The two groups had a significant differences with very high correlation coefficient (P<0.01) in postoperative period (Figure 1).

Electrophysiological Evaluation

The results of the electrophysiological analysis are shown in Table 1.

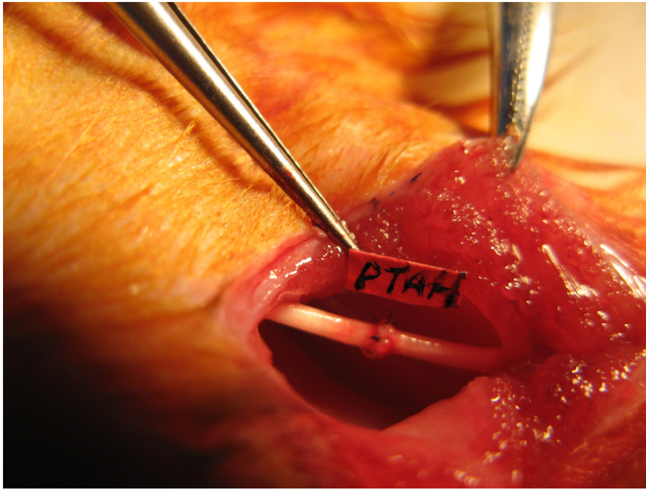

Figure 2. Macroscopic appearance of repaired sciatic nerve with epineurial nylon sutures in operation.

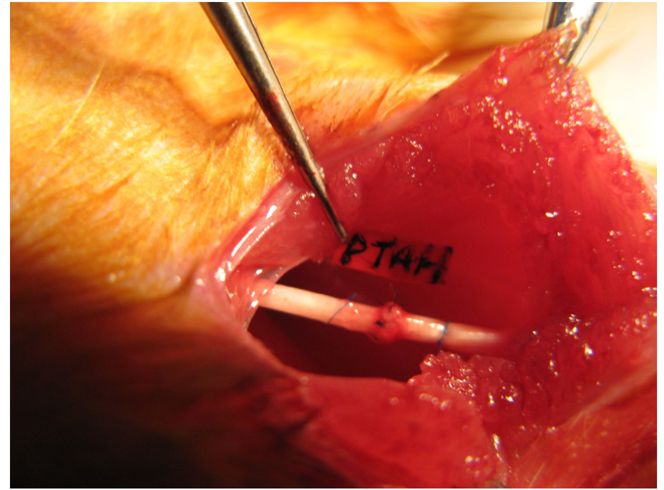

Figure 3. 20-mm length of the sciatic nerve was removed for PTAH staining at 12 weeks after operation.

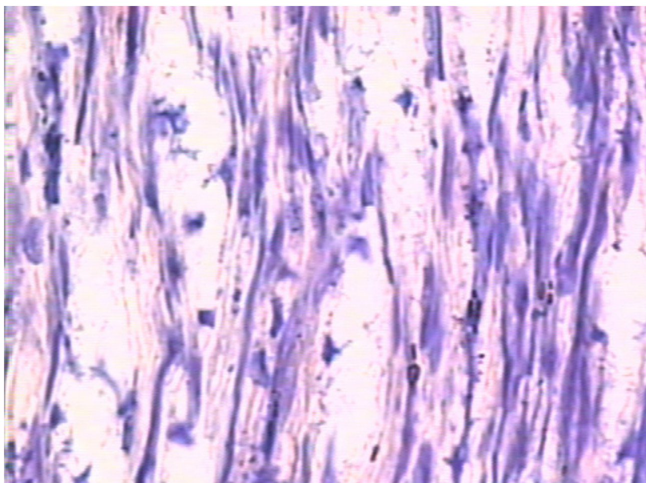

Figure 4. LM findings in specimen stained with PTAH in VPA group (×400).

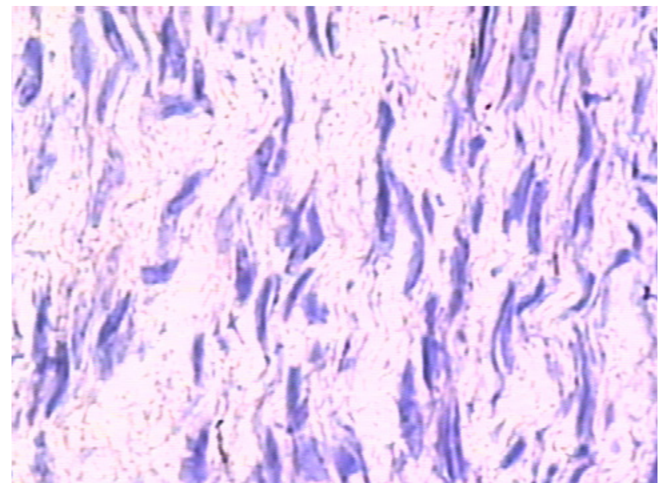

Figure 5. LM findings in specimen stained with PTAH in control group (×400).

Macroscopic Findings

Shortly after the electrophysiological evaluation, a 20-mm length of the sciatic nerve was respectively removed for PTAH staining (5 mm distal to the repair site, 5 mm proximal to the repair site) (Figure 2, 3) and methylene blue-basic fuchsin staining.(the left part of removed nerve). No disruption of sciatic nerve was observed in any animals, and not much adherent scar tissue around the repair site of nerve was observed in VPA group. However, a large quantity of scar tissue was formed around the repair site of sciatic nerve in control group.

Histological Observation

Observation of nerve by PTAH staining

Histochemical staining of longitude-sections of rat sciatic nerve with the method of PTAH is a very efficient method for observing successiveness of nerve. Under the LM (Figure 4, 5), the nerve fiber was stained blue, collagen fiber and reticular fiber were stained brown red, meanwhile, the cytoplasm was stained shallow brown red. The nerve fibers in VPA group arranged in order and shaped regularly. It indicated that few of VPA group presented necrosis and successiveness of nerve fiber were obviously better than that in control group. The nerve fibers in control group were easily found arranging confusedly and the successiveness interrupt. Moreover, the normal frame of nerve in the necrosis area could not be distinguished.

Observation of nerve by methylene blue-basic fuchsin staining

LM observation There was no statistical difference between the sizes of individual myelinated nerve fibers in rats of VAP group and control group. Meanwhile, there was no difference in morphological appearance between proximal specimens harvested from each group as well. (Figure 6, 7). Under the oil microscopy(Figure 8, 12), a large quantity of thick myelinated nerve fibers

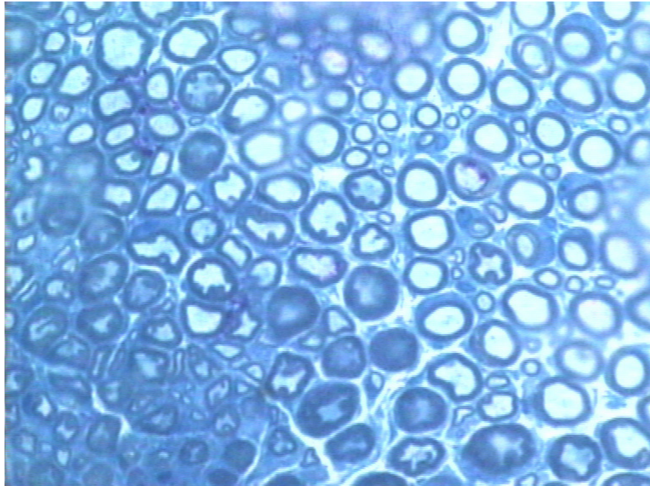

Figure 6. Proximal specimen, 0.5 cm to repaired site in VPA group (×400).

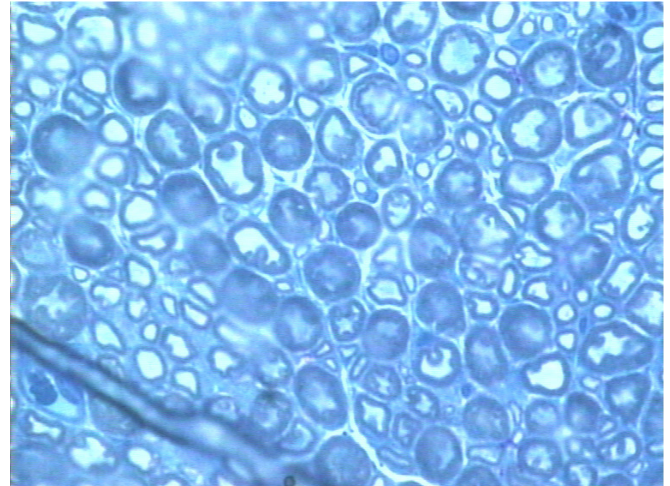

Figure 7. Proximal specimen, 0.5 cm to repaired site in control group (×400).

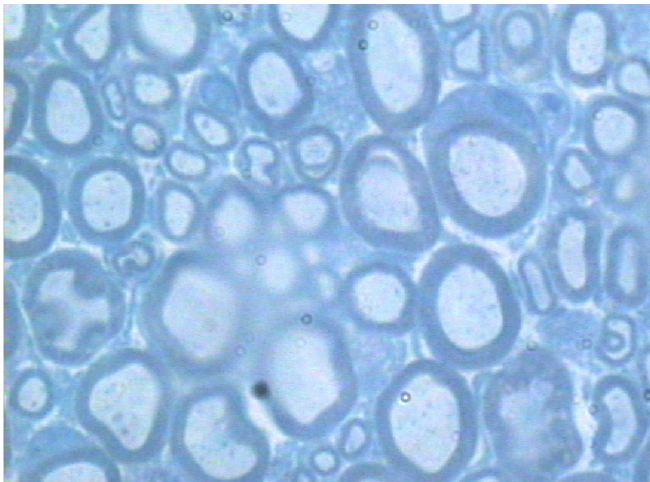

Figure 8. Proximal specimen, 0.5 cm to repaired site in VPA group (×1000,oil microscopy).

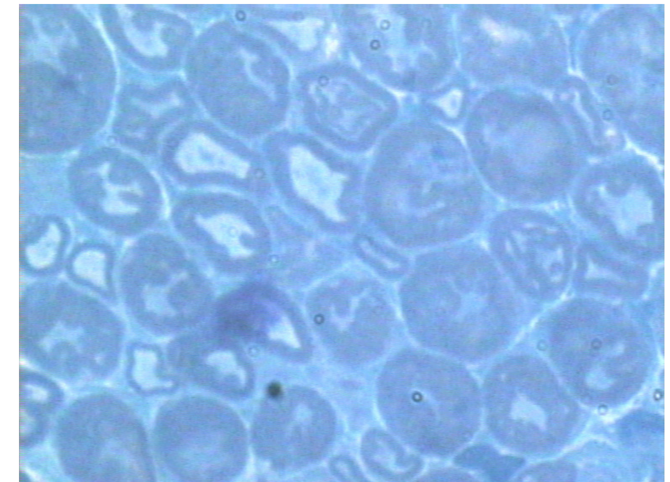

Figure 9. Proximal specimen, 0.5 cm to repaired site in control group (×1000,oil microscopy).

were packed in nerve bundles over cross sections from specimens harvested from the VAP group. Axons in the endoneurium were defined by their surrounding myelin sheaths, stained blue-black by the methylene blue. However, in the control group, few myelinated nerve fibers posess intact normal outline, which present the sign of denaturalization (Figure 9, 13). The comparatively high density of well-myelinated fibers occurred in both the proximal and distal specimens (Figure 6, 10). In the control group, few myelinated nerve fibers could be found in the distal specimens, shaping irregularly as well (Figure 11, 13).

TEM observation. TEM findings in Figure 14 shows a distal specimen 0.7 cm to the tip of the sciatic nerve in the VPA group; and in Figure 15, a distal specimen 0.7 cm to the tip of the sciatic nerve in the control group at 12 weeks after operation. Furthermore, the demyelination of myelinated nerve fibers was obviously observed in control group, showing the necrosis of nerve as well.

Morphometrical Analysis

There was no statistical difference between both groups when comparing

the thickness of myelin sheaths of specimens harvested from proximal segments ($P > 0.05$). However, regenerated nerve fibers showed a higher density and more mature myelinization when the distal specimens were observed in the VPA group, indicating a better regeneration (Table 2).

Discussion

It has been widely accepted that peripheral nerve injury is a difficult problem to solve clinically (19). Uptodate, there was not miracle drug to conquer it. It has recently reported that VPA robustly promotes neurite outgrowth and activates the ERK mitigen-activated protein kinase pathway, a signaling pathway utilized by many endogenous neurotrophic factors (20-23). It was

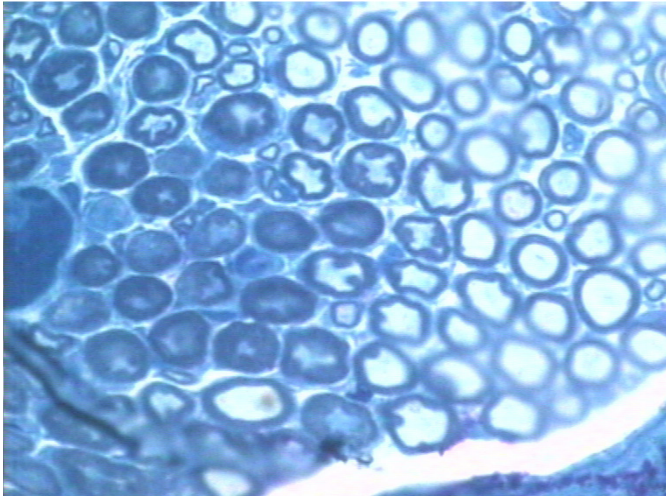

Figure 10. Distal specimen, 0.5 cm to repaired site in VPA group (×400).

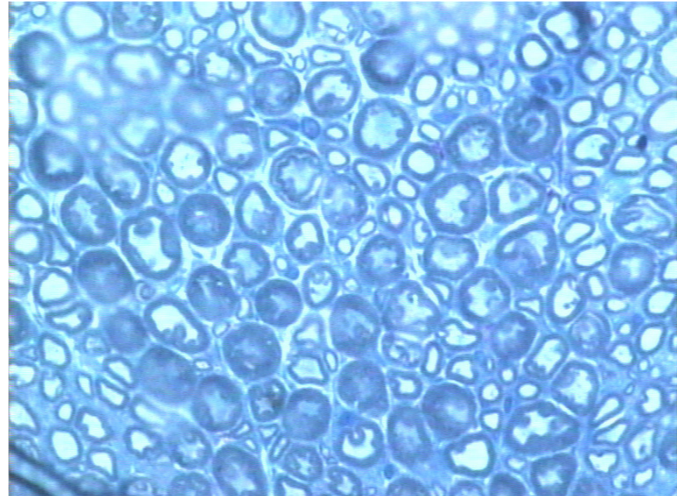

Figure11. Distal specimen, 0.5 cm to repaired site in control group (×400).

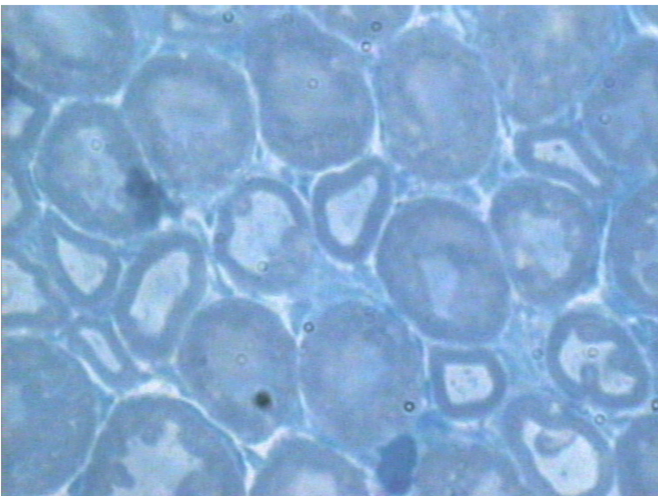

Figure 12. Distal specimen, 0.5 cm to repaired site in VPA group (×1000,oil microscopy).

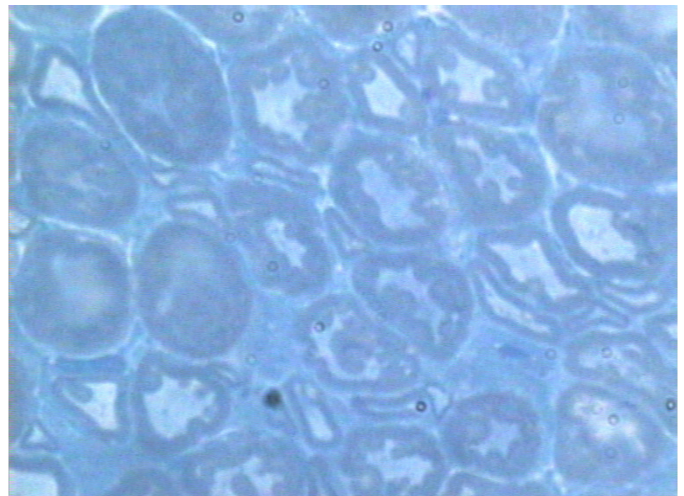

Figure 13. Distal specimen, 0.5 cm to repaired sit in control group (×1000, oil microscopy).

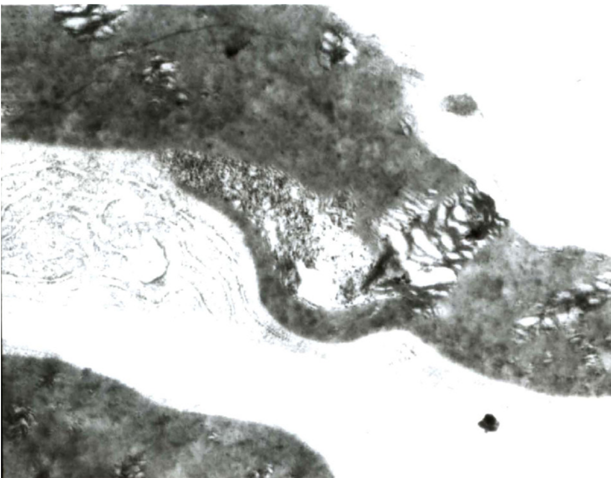

Figure14. TEM findings of myelinated nerve fiber distal segment of sciatic nerve in VPA group.

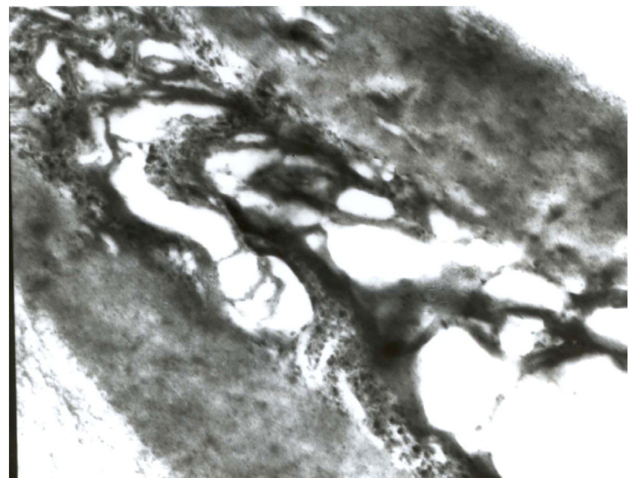

Figure 15. TEM findings of myelinated nerve fiber in distal segment of sciatic nerve in control group.

Table 2. Results of Morphometric Analysis Results at 12 Weeks ($\bar{x}$ ±s, n=15)

Group	Proximal		Distal	
	Number	Thickness(μm)	Number	Thickness(μm)
VPA	287±7.19	0.78±0.05	243±5.90	0.40±0.06
Control	240±7.67	0.76±0.05	211±7.57	0.29±0.05

VPA group vs control group, *P*<0.05 *

note worthy that VPA have recently been demonstrated to increase the expression of the cytoprotective protein bcl-2 in the central nervous system and human neuroblastoma SH-SY5Y cells (24). In the present research, functional observation, electrophysiological measurement, and a morphometrical study were carried out to evaluate the quality of newly regenerated nerves. Moreover, we can find better results in the VAP group when SFI, MCV, axon number, and thickness of myelin sheath were taken into consideration. These results demonstrate that VPA is able to improve sciatic nerve regeneration by increasing the number of regenerated myelinated nerve fibers in rats.

In the present study, we have shown that VPA at therapeutic concentration produces effects similar to those of neurotrophic factors, namely activation of the ERK pathway and promotion of neurite growth and cell survival. VPA slao increases GAP-43 and Bcl-2 levels and enhances the neuronal function (25). The precise mechanisms by which VPA brings about these effects are currently unknown, and they likely involve the activation of intracellular signaling pathways. In vitro, Yuan *et al* had demonstrated it. It is possible that the effect is produced by the direct activation of ERK signal transduction pathway by VPA, or indirect activation of ERK pathway following enhanced synthesis of neurotrophins by VPA (3). It is because VPA at clinically therapeutic dosage can produce effects similar to those of neurotrophins by activation of the ERK pathway and increase the expression of bcl-2 and growth cone-associated protein 43. Moreover, it is a quite important reason that VPA possesses the natural characteristic of penetrating rapidly through the blood-brain barrier (BBB) because of its small molecular weight (10, 11). The present study has demonstrated that VPA has the protective effect on motor neuron of spinal cord in rats. As well known as, doctors are used to applying neurotrophins locally around the lesion of injuried nerve in clinic. The neurotrophins has the obvious effect that promote neurite growth in vitro, but it has not the same effect as in vivo yet. It is possible that neurotrophins are degraded by enzymatic in the blood and difficult to pass through the BBB (2).

Conclusions

We conclude that VPA can significantly increase the number of regenerated myelinated nerve fibers in rats. Moreover, VPA possesses the natural characteristic of penetrating rapidly through the BBB and has been safely used clinically as an effective anti-epileptic drug and mood stabilizer for many years. And the low price provide the amplitude foreground for VPA using as well. Therefore, VPA is hopeful to be applied for the patients suffering peripheral nerve injury in the future.

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