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4-AP-3-MeOH Promotes Structural and Functional Spontaneous Recovery in the Acute Sciatic Nerve Stretch Injury

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Abstract

Background: 4-AP-3-MeOH, a derivative of 4-aminopyridine, was developed and demonstrated to prevent nerve pulse diffusion due to myelin damage and significantly enhance axonal conduction following nerve injury. Currently, repurposing the existing drug such as 4-AP-3-MeOH to restore motor function is a promising and potential therapy of peripheral nerve injury. However, to evaluate drug effect on sciatic nerve injury is full of challenge.

Methods: Sciatic functional index was used to determine and measure the walking track in the stretch injury model. Nerve conductivity was performed by electrical stimulation of a nerve and recording the compound muscle action potential. Myelin thickness and regeneration was imaged and measured with transmission electron microscopy (TEM).

Results: In this study, we developed a sciatic nerve injury model to minimize the spontaneous recovery mechanism and found that 4-AP-3-MeOH not only improved walking ability of the animals but also reduced the sensitivity to thermal stimulus. More interesting, 4-AP-3-MeOH enhanced and recovered electric conductivity of injured nerve; our TEM results indicated that the axon sheath thickness was increased and myelin was regenerated, which was an important evidence to support the recovery of injured nerve conductivity with 4-AP-3-MeOH treatment.

Conclusions: In summary, our studies suggest that 4-AP-3-MeOH is a viable and promising approach to the therapy of peripheral nerve injury and in support of repurposing the existing drug to restore motor function.

Keywords
peripheral nerve injury, sciatic nerve injury, 4-AP-3-MeOH, myelin regeneration

Background

Peripheral nerve injury is a common and major clinical disease without effective treatment and its recovery process is very complicated and very hard to predict. Nerve stretch injury, a common and special type of acute peripheral nerve injury, sometimes is spontaneously recovered without any external treatment if not all nerves are transected. However, long-term or serious denervation causes dysfunction and degeneration of muscle deteriorated and irreversible even pulse transmission along the injured axon is still continuous. So it is critical to apply surgical or drug interruption at the specific time window to recover the function of motor neuron. Sometimes surgical interruption is performed too early and makes the spontaneous recovery opportunity lost. Most time, therapy effect of drug interruption coincides with the spontaneous recovery together. For drug interruption, it is crucial and urgent to determine injury tolerance, if beyond it, the spontaneous recovery is impossible, and see whether medicine interruptions, especially for the existing therapeutic drug, can be repurposed and used to shorten the duration for nerve injury recovery.

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Some medicines have been clinically applied to cure peripheral nerve injury, such as basic fibroblast growth factor, nerve growth factor, Mecobalamin, and vitamin B, but all these medicines act slowly and need long term to be effective.[1,2] 4-Aminopyridine (4-AP), one of US Food and Drug Administration (FDA) approval drug, is a small molecule potassium channel blocker and has been proved to ameliorate motor function and walking ability in the patient with multiple sclerosis (MS)[3-5]; however, only the concentration of 4-AP above 100 μM can improve the recovery of nerve transduction dysfunction,[6] the maximum tolerance of 4-AP concentration in human blood is 0.5 to 1 μM, otherwise this inhibits the respiration and causes some side effects such as seizure and anxiety.[7]

4-AP-3-MeOH, a derivative of 4-AP, was developed by Gary group[8] and demonstrated to block potassium channels and restore impulse conduction as well as prevent nerve pulse diffusion due to myelin damage and significantly enhance axonal conduction following nerve injury. More importantly, 4-AP-3-MeOH can repair the transduction of injured nerve without the side effect and has the wide range of the effective concentration window. It is reported that the minimum effective concentration of 4-AP-3-MeOH is 0.1 μM.[6-9] Some in vitro studies also indicate that 4-AP-3-MeOH can be used to restore the function of nerve injury via myelin regeneration,[9,10] but all these therapy effects cannot be discriminated from drug effect or spontaneous recovery effect with the significant variance itself. Although 4-AP has provided a new potential therapy for durable recovery and demyelination in acute peripheral nerve injury in the animal,[10] all these functional improvements of 4-AP-3-MeOH on nerve injury recovery are still limited in vitro; in the present study, we create an animal model of nerve stretch recovery to minimize therapy effect of the spontaneous recovery and repurpose 4-AP-3-MeOH to treat nerve stretch injury and investigate the therapy effect of 4-AP-3-MeOH on the recovery of nerve stretch injury without the spontaneous recovery in the animal.

Materials and Methods

Animals

Twenty-four adult male Sprague Dawley (SD) rats (200-300 g) were housed in pathogen-free animal facility with a 12:12-hour light/dark cycle and had free access to food and water. All SD rats were obtained from Tonji Medical College Experimental Animal Department. All animal tests were carried out in accordance with the US National Institute of Health (NIH) Guide for the Care and Use of Laboratory. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Affiliated Puai Hospital of Tongji Medical College of Huazhong University of Science and Technology.

Stretch Injury Model

The animals were anesthetized with isoflurane inhalation or the mixture of ketamine (90-120 mg/kg) and xylazine (10 mg/kg); dexamethasone and buprenorphine were subcutaneously administered to reduce inflammation and pain. These rats were divided into control (group A), mild (group B), moderate (group C), and severe traction groups (group D), with 6 rats for each group. When the animal was total anesthetized, it was put on the experiment table and the sciatic nerve on the left side was exposed. The fur along the length of femur was removed and the lateral skin incision site was made to expose the sciatic nerve posterior to the femur. And then the sciatic nerve was bluntly separated. An injury device with a different stretch magnitude (1.0°N, 1.8°N, and 3.6°N) was used to pull sciatic nerve for 20 minutes to generate the different nerve injury group (control, mild, moderate, and severe), respectively. Buprenorphine (0.2 mg/kg) was given every day to relieve postoperative analgesia on the day of surgery until the animals were totally recovered.

The animal in moderate group was divided into 2 subgroups: saline treatment and 4-AP-MeOH treatment. Saline and 4-AP-MeOH solution were systemically administrated (10 μg/d, intraperitoneally) to treat the animal with nerve stretch injury during the duration of experiment, respectively.[10]

All surgery process described above was performed at the sterile environment and approved by IACUC in Affiliated Puai Hospital of Tongji Medical College of Huazhong University of Science and Technology.

von Frey and Hargreaves Test

Mechanical allodynia and thermal hyperalgesia were evaluated using the von Frey and Hargreaves test, respectively, as described by Tseng et al.[11] Briefly, we performed these assessments on the day before surgery for a baseline, followed by postoperation on the different days (3, 5, 14, and 21). Rats were placed on the respective apparatus and allowed to habituate for 15 minutes. Although one investigator controlled the nociceptive stimulus, the other investigator recorded the values from the response of the hind paw associated with discomfort (hind paw retraction, licking, shaking, flinching, 4-paw jumping following stimulation).

Sciatic Function Index Measurement

As described by de Medinaceli et al,[10,12,13] the sciatic functional index (SFI) was used to determine and measure the walking track. Briefly, prior to rat walking, a 50-cm narrow corridor lined with paper. To obtain footprints, hind paws were placed on an ink blotter and the animals were placed on a white piece of paper. Both feet produced 5 to 6 prints. Rats were tested weekly over the course of the experiment. Surgery was done on the left sciatic nerve of each animal and the right hind limb was used as internal control. Footprints were collected from the experimental (E, left) and normal (N, right) sides. Prints were measured for the following parameters: distance between foot prints (TOF), the entire plantar length (PL), the distance from the first to fifth toes, the toe spread (TS), the distance between the second and fourth toes, and the

intermediary toe spread (IT). The SFI was calculated according to the following formula:

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

where E is the injured limb and N is the control limb.[13] The scores of SFI from this formula range from 0 to −100. Zero describes normal function and −100 complete transection of the sciatic nerve.

Assessment of Neuropathic Pain

Mechanical allodynia and thermal hyperalgesia were developed and evaluated using the von Frey and Hargreaves test, respectively.[10,14] Here, we define the assessments on day 0 (presurgery) as the baseline, followed by postoperatively on days 7, 14, and 21. Prior to each testing round, rats were allowed to habituate for 15 minutes on the respective apparatus. Each experiment was performed by 2 investigators, with one controlling the nociceptive stimulus while the other recorded the values from the response of the hind paw associated with discomfort (hind paw retraction, licking, shaking, flinching, 4-paw jumping following stimulation).

Transmission Electron Microscopy

The animals were deeply anesthetized with ketamine/xylazine and perfused transcardially with 0.9% saline followed by ice-cold 4% paraformaldehyde in 0.14 M phosphate buffer (pH 7.4). Sciatic nerves were carefully isolated and immersion fixed overnight in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer solution. The nerves were rinsed in 0.1 M sodium cacodylate buffer solutions and post-fixed for 2 hours in 2.0% osmium tetroxide combined with 0.2 M cacodylate buffer. After rinsing in distilled water, the sections were dehydrated in a graded series of ethanol (50%, 70%, and 95% for 15 minutes, respectively) and 100% ethanol for 3 times, transitioned into propylene oxide followed by Epon/Araldite epoxy resin overnight, and finally embedded and polymerized at 60°C for 48 hours. Using an ultramicrotome and a diamond knife, sections of 50-μm thickness were collected onto metal mesh grids and stained with uranyl acetate and lead citrate. The stained grids were examined and photographed using Zeiss-EM902 transmission electron microscopy (TEM).

Compound Action Potential Recording

Nerve conduction studies were performed by electrical stimulation of a nerve and recording the compound muscle action potential (CMAP) from needle electrodes overlying a muscle supplied by that nerve, as described previously.[15,16] Briefly, electromyogram recording was performed with subdermal stainless steel needle electrode placed into the hind limbs (6 V, 0.1 milliseconds, 1 Hz, 5-15 mA). The stimulating electrode was placed in resting muscle on gluteal fold to obtain the first CMAP. Then the stimulating electrode was moved to popliteal fossa with a 10-mm fixed distance from gluteal fold to get the second record of CMAP.

Image Analysis

Image of cross-sectioned sciatic nerve taken by TEM was processed by Fiji software (version fiji 1.5.2; NIH, Bethesda, MA) and Matlab software (version 2015a; MathWorks Inc., Natick, MA) to measure myelin thickness and area. To analyze myelin thickness and area, 10 randomly chosen axons were chosen for each mouse and at least 3 thickness measurements on the same axon were averaged to reduce measurement deviation. Data are presented as means $\pm$ standard error. Statistical evaluations were based on 2-tailed t test and χ^2 test (GraphPad Prism7; criterion, $P < .05$).

Result

Develop Stretch Injury Animal Model and Validate the Recovery Duration for the Different Types of Injury

Restoration of motor function is the primary goal in the treatment of peripheral nerve injury. Repurposing the drug to restore motor function is very beneficial to the therapy of peripheral nerve injury; however, the different types of injury has the different effect of spontaneous recovery. To address whether the therapy effect is from the repurposed drug, while excluding the effect of its spontaneous recovery, we generate and build the type of sciatic injury group to limit the spontaneous recovery and test the therapy effect of the drug.

To avoid prejudice experiments toward the nerve regeneration, we introduced a standard model of sciatic nerve injury to make the different injury with the different stretch strength.[13,17–19] All the injury was divided into 4 groups based on the stretch strength: control, mild, moderate, and severe. Motor function was assessed with standard SFI and compound action potential (CAP) described in Method parts. Our SFI measurement indicated that compared to the control group, 2 weeks after nerve injury, severe group totally lose nerve function and nerve function in moderate group has the significant difference. In mild group, nerve function fully recovered (Figure 1A-C). In order to investigate whether the decrease or loss of nerve function was caused by nerve conductivity, we performed CAP experiment to measure CAP 5 days after nerve injury. We found that the conductivity in severe injury group was totally blocked. Compared to the control group, action potential in moderate injury group decreased before 20 days after the injury and began to recover 20 days after the injury (Figure 1D).

Our results indicated that the spontaneous nerve injury recovery was dependent on the injury type caused by the different stretch strength. Comparing nerve function loss among these 3 groups, severe group totally lost nerve function and could not recover, moderate injury group could not totally recover, and nerve function continued to deteriorate within 2 weeks after nerve injury. Amplitude in mild group has no

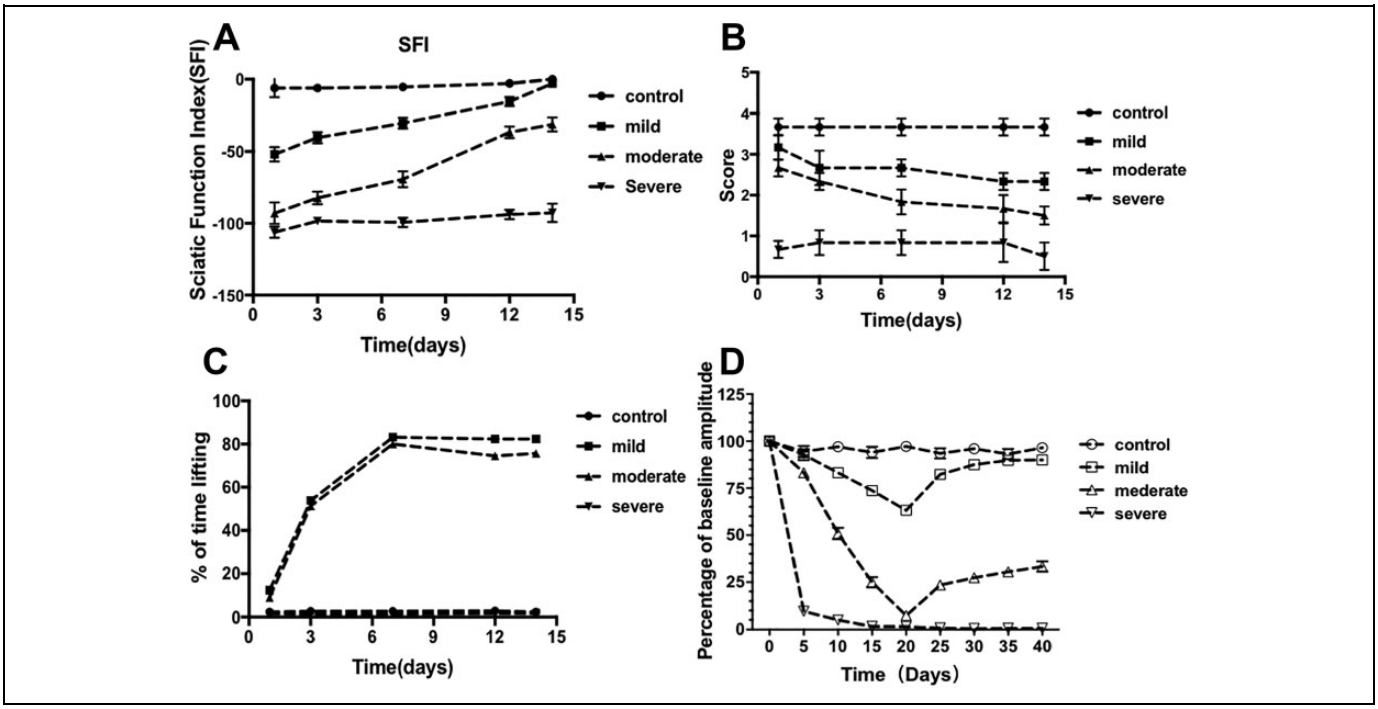

Figure 1. Nerve function measurement and evaluation after injury with the different stretch strength. A, Measure sciatic function at the different injury condition. B, Rotarod test score at the different injury. C, The sensitivity to thermal stimuli at the different injury model. D, Compound action potential (CAP) measurement at the different injury condition (each group: n = 6).

significant difference (Figure 1). To avoid the effect of spontaneous recovery on drug therapy, here we choose moderate injury model that minimizes therapy effect from the spontaneous recovery and measures the effect of 4-AP-3-MEOH on the recovery of sciatic nerve injury.

4-AP-3-MEOH Administration Improves the Recovery of Nerve Conduction and Walking Capability

To determine whether the restoration of motor function was mainly dependent on the presence of drug effect, we select moderate injury model as treatment target and all the measurements were performed 24 hours after treatment with 4-AP-3-MeOH. We first investigated the effect of drug on the potential treatment of nerve injury and found that the improvement in walking ability significantly began 3 days after daily treatment and last the whole treatment; 15 days after treatment, nerve function was very close to the normal level (Figure 2A and B). To further validate the therapy effect of drug on motion ability, we examine the movement of the animal with RotaRod performance test, our results indicated that the decline tendency of nerve function loss was prevented at day 12 and day 15 after the initiation of 4-AP-3-MeOH treatments, there were some significant difference of the effect of movement before treatment and nontreatment group (Figure 2C and D).

Although we introduce the standard model to make sciatic nerve stretch, peripheral nerve damage always comes with neuropathic pain syndromes. To address the effect of 4-AP-3-MeOH on pain relief from sciatic nerve stretch, we daily systematical administrate saline and 4-AP-MeOH to treat these animals; the animal with injury surgery was examined for thermal and mechanical stimulus at 0, 7, and 14 days postinjury. Saline-treated rat showed increasing sensitivity 3 days after initiation of the surgery, while rat treated with 4-AP-3-MeOH decreases the sensitivity to thermal and mechanical stimulus (Figure 3A and B), respectively.

Daily 4-AP-MeOH Administrations Ameliorate Nerve Conduction via Myelin Regeneration

Our current study indicates that 4-AP-3-MeOH treatments enhance and improve walking ability of the animals with moderate nerve injury. We hypothesized that this nerve function improvement was related to nerve conductivity recovery and myelin regeneration. To address this possibility, we introduced CAP recording system to measure the CAP 24 hours after daily treatment. All measurements were performed at the same condition during the different days. In saline-treated group, CAP amplitude decreased from day 0 to day 20, and CAP began to recover after 20 days postinjury, only partial recovery to 30% normal level. Although 4-AP-3-MeOH reduces and delays nerve dysfunction, by 20 days postinjury, CAP recovered to 30% normal level 40 days after treatment followed by 50% at day 30 and 60% at day 40 (Figure 4A and B), respectively, all of which improvements were significant compared to saline-treated group.

To address whether nerve function improvement was related to morphological change of axon structure, including myelination and myelin cross-section area, we use TEM to investigate

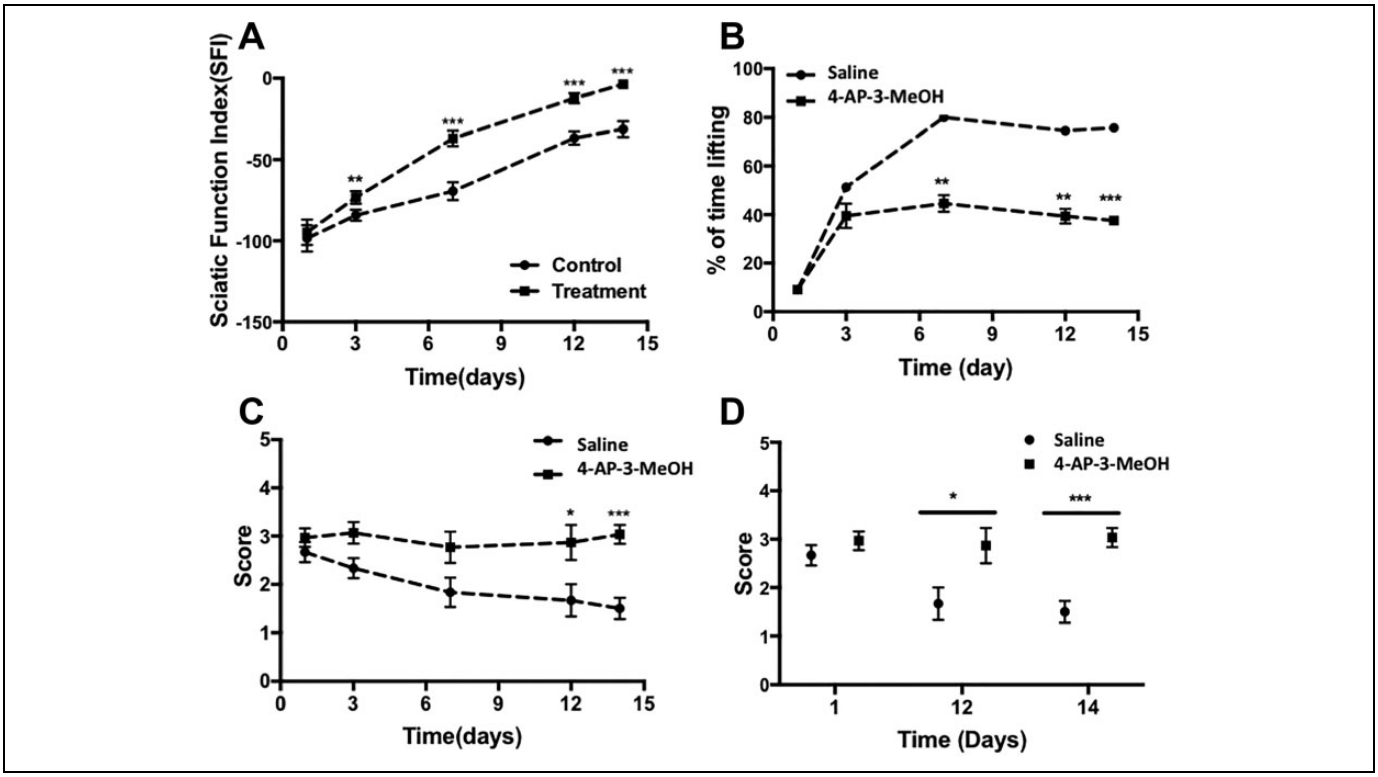

Figure 2. 4-AP-3-MeOH treatment improves functional recovery of sciatic nerve injury. A, 4-AP-3-MeOH enhanced the recovery of sciatic nerve function compared with saline treatment from 3 to 15 days after injury (n = 6, 1-way ANOVA analysis: $P < .05$). B, 4-AP-3-MeOH treatment reduced the sensitivity of sciatic nerve to thermal stimuli compared with saline treatment from 3 to 15 days after injury (n = 6, 2-way ANOVA analysis: $P < .05$). C, 4-AP-3-MeOH treatment improved running ability on Rotarod after sciatic nerve injury compared with saline treatment from 3 to 15 days after injury and significant therapy effect occurred 12 days after the treatment. D, Compare running test score on Rotarod between 4-AP-3-MeOH and saline treatment at day 1, day 12, and day 14 after injury (n = 6, 1-way ANOVA analysis, $P < .05$). ANOVA indicates analysis of variance.

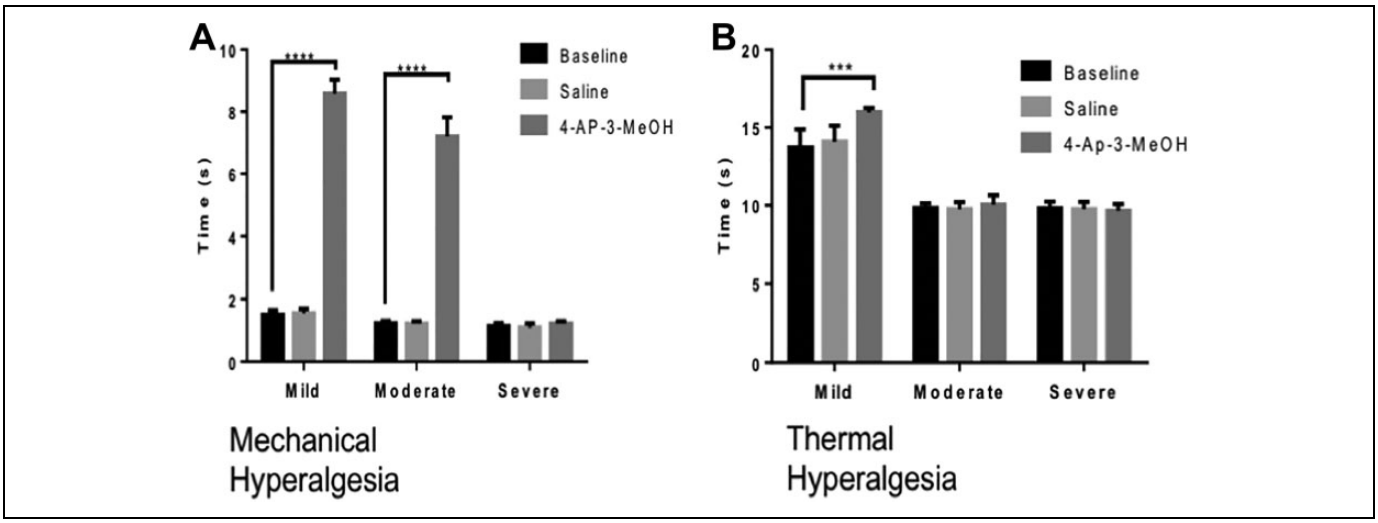

Figure 3. Effect of 4-AP-3-MeOH on the acute sciatic nerve stretch injury during mechanical hyperalgesia and thermal hyperalgesia test. A, During mechanical hyperalgesia test, 4-AP-3-MeOH significantly recovers the response of mild and moderate injured sciatic nerve to mechanical stimulus. B, During thermal hyperalgesia test, 4-AP-3-MeOH significantly restores the response of mild injured sciatic nerve to thermal stimulus.

axon structure change caused by 4-AP-MeOH treatments. Ultrastructure analysis showed that axonal cross-sectional area in nerves with 4-AP-3-MeOH treatments was significantly increased than that with saline treatment (Figure 5A). In the control group (axon without any injury), the average area of axon was $0.4 \pm 0.06\ mm^2$ and the thickness of axon sheath was

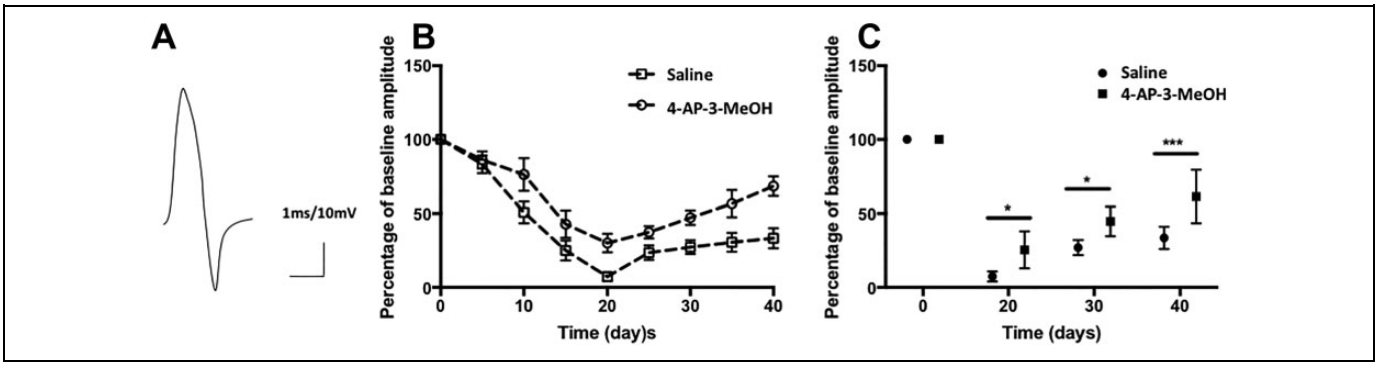

Figure 4. 4-AP-3-MeOH treatment improves nerve conduction of sciatic nerve injury. A, The representative recording of sciatic compound muscle action potential in an adult SD rats (6 months old). B, 4-AP-3-MeOH enhanced nerve conduction compared with saline treatment from 20 to 40 days compared with saline treatment. C, 4-AP-3-MeOH enhanced nerve conduction compared with saline treatment at day 20, day 30, and day 40 compared with saline treatment (n = 6, 1-way ANOVA analysis). ANOVA indicates analysis of variance.

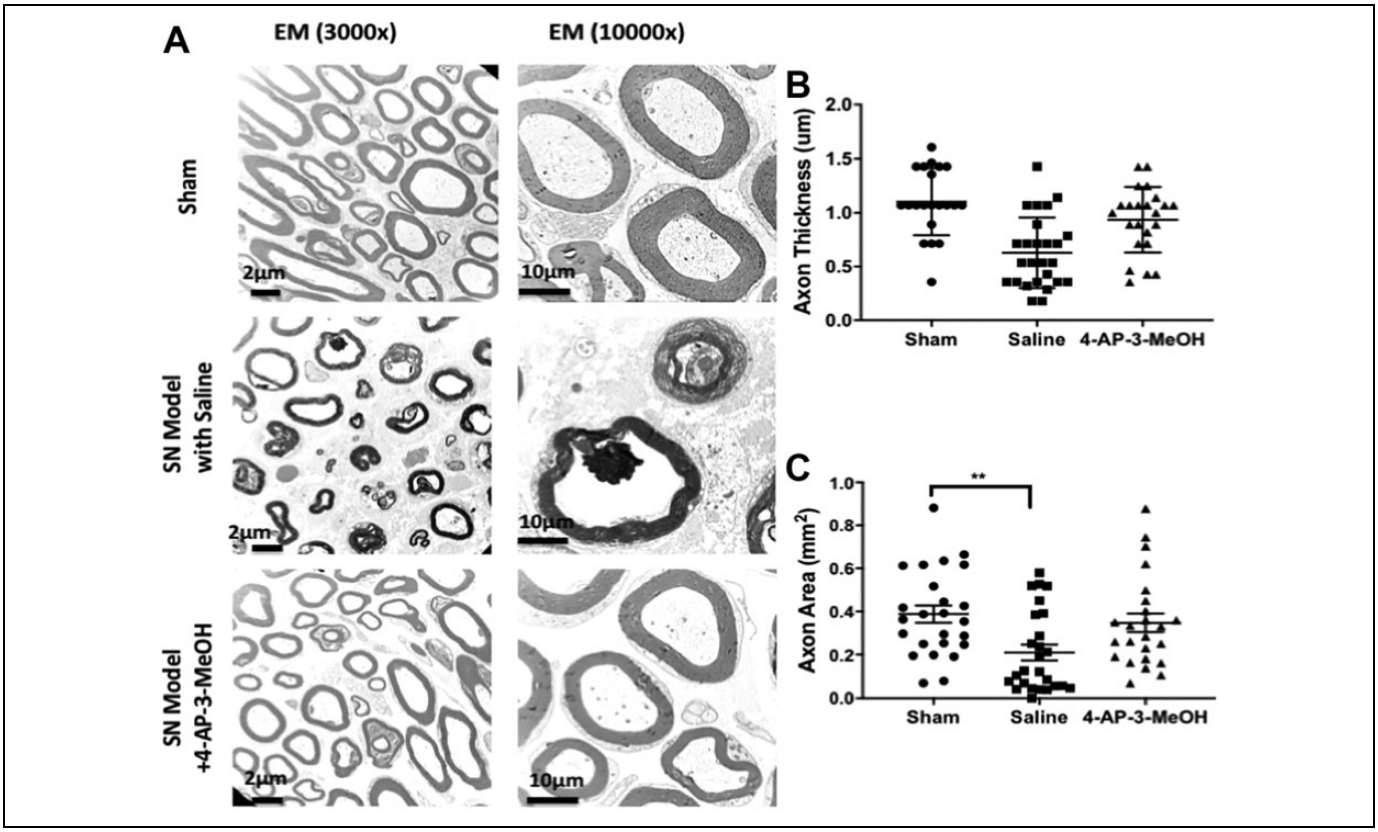

Figure 5. 4-AP-3-MeOH treatment increases axon cross-sectional area and myelin sheath thickness. A, Electron microscopy image of sham, saline, and 4-AP-3-MeOH treatments. B, Axon thickness between sham, saline, and 4-AP-3-MeOH treatments (n = 4, 1-way analysis, $P < .05$). C, Axon cross-sectional area between control, injury, and treatment (n = 4, 1-way ANOVA analysis, $P < .05$). ANOVA indicates analysis of variance.

1.1 ± 0.3 μM; in injured axon with saline treatment, the average area of axon was 0.2 ± 0.02 mm^2, with 0.6 ± 0.15 μM thickness of axon sheath; in injured axon with 4-AP-3-MeOH treatment, the average area of axon was restored to 0.38 ± 0.02 mm^2, with 0.8 ± 0.25 μM thickness of axon sheath (Figure 5B and C). The ratio of the inner axonal diameter to the total outer diameter (G ratio), widely used as a functional and structural index of optimal axonal myelination, was calculated to evaluate the effect of 4-AP-3-MeOH on myelin regeneration. We found that G ration decreased from 0.736 ± 0.049 to 0.662 ± 0.050, very close to the value (0.633 ± 0.044) in the control group. So all these results revealed that 4-AP-3-MeOH prompted myelin regeneration along the axons.

Discussion

The development of a brand-new drug provides unprecedented opportunities for some neuronal disease, but this is the long-

term and high money and effort cost process due to bottlenecks and barriers in the therapeutic development. So it is pivotal to develop the strategy to reduce time frame, decrease costs, and improve success rates. Repurposing the existing drugs for the novel application is the optimal and promising strategy and opportunity of efficient therapy development for some diseases because these agents have been approved by US FDA and used in the previous clinic. If these drugs are tested efficiently for other disease, they could be ready for clinical trial quickly. 4-Aminopyridine, a small molecule potassium channel blocker, is the first drug aiming to improve ambulatory impairment in the treatment of MS.[5] And 4-AP is demonstrated to protect never conduction by stabilizing the membrane potential of the axon and blocking exposed potassium channels in demyelinated fibers, which indicates it is a potential and promising effective therapy in peripheral nerve injury. However, some evidences indicate that 4-AP, potassium ion channel blocker, can be used to restore nerve function; its side effect limits its clinic application, for example, the minimum concentration of 4-AP in blood to restore nerve function is 100 μM[8]; unfortunately, the maximum concentration in blood human can endure is 0.5 to 1 μM, and the high concentration beyond this limitation will cause respiration disturbance and epileptic seizure occur. To overcome this limitation, 4-AP-3-MeoH, a derivative of 4-AP, was developed to investigate the possibility of potential application to peripheral nerve injury in the animal or clinic.[19]

Here, we aim to demonstrate and validate the therapy of an animal model with sciatic nerve injury with 4-AP-3-MeOH (the derivative of 4-AP) treatments. Some studies indicated that 4-AP-3-MeOH, a fast potassium ion channel inhibitor, restored the nerve transduction for the injured nerve in guinea pig and reduced the nerve transduction for the recovery of CAP in MS model of the rat.[19] Leung et al[8] demonstrated that 4-AP-3-MeOH restored CAP and the transduction of injured nerve without inducing multiple responses from multiple stimuli in MS rat model.

Some in vitro studies[9,11] from CAP measurement indicated concentration-dependent effect and wide secure window of the application of 4-AP-3-MeOH: the minimum effective concentration is 0.1 μM, CAP increase with its concentration increasing from 1 to 100 μM, but when the concentration reaches 1 mM, CAP begins to decrease and has no therapy effect; furthermore, high concentration such as 10 mM inhibited CAP generation and the normal nerve transduction. So, in our study, we take 100 μM as 4-AP-3-MeOH treatment concentration and validate its potential therapy effect on sciatic injury in the animal.[19]

Some studies indicate that 4-AP-3-MeOH can cause functional recovery and axon myelination after injury in MS model; little is known about the mechanism of the therapy effect of 4-AP-3-MeOH on the sciatic nerve injury. As we know, spontaneous recovery always accompanies with sciatic injury and its variation is the significant difference among the different types of sciatic injury. In this study, the critical point, where nerve function almost cannot be spontaneously restored without any external treatment, was determined to develop sciatic injury model and investigate the effect of drug treatment effect, while excluding the therapy effect from spontaneous recovery mechanism. Even under this condition, we found that 4-AP-3-MeOH restore nerve function after injury and recover animal behavior with myelin regeneration and thickness increase.

Conclusion

In summary, our studies indicated that 4-AP-3-MeOH significantly restores nerve function after injury with myelin regeneration and axon sheath thickness increasing; our study repurposes the potential and promising application of 4-AP-3-MeOH for the therapy of peripheral nerve injury.

Authors' Note

Y.C., W.W., and D.X. conceived the study concept and designed the experiment and data analysis. Y.C., W.W., Z.Z., and D.R. carried out the experiments. All the authors wrote the manuscript. Y.C. and W.W. contributed equally.The data sets used and analyzed during the current study are available from the corresponding author on reasonable request. The animal study proposal was approved by the Institutional Animal Care and Use Committee (IACUC) of Affiliated Puai Hospital of Tongji Medical College of Huazhong University of Science and Technology. All animal tests were carried out in accordance with the US National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals published by the US National Academy of Sciences (http://oacu.od.nih.gov/regs/index.htm).

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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