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ORIGINAL ARTICLE

Nicotine exerts neuroprotective effects by attenuating local inflammatory cytokine production following crush injury to rat sciatic nerves

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ABSTRACT. ***Background*: Recent studies have demonstrated that nicotine exhibited anti-inflammatory and neuroprotective properties by interacting with the alpha 7 nicotinic acetylcholine receptor (α7nAChR). However, the role of nicotine in regeneration during peripheral nerve injury has not been elucidated. The aim of this study was to investigate whether nicotine down-regulated production of proinflammatory cytokines and promoted peripheral nerve regeneration in rats.** ***Methods*: Rats challenged with sciatic nerve crush injury were treated with nicotine (1.5 mg/kg), three times per day. The expression of the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin (IL-1β), pinch test results, growth-associated protein 43 (GAP-43) expression, morphometric analyses, and the sciatic functional indexes were determined in sciatic nerves.** ***Results*: Treatment with nicotine decreased local levels of TNF-α and IL-1β, and increased the expression of GAP-43. Nicotine also improved nerve regeneration and functional recovery. The overall protective effects of nicotine were reversed by concomitant treatment with α7nACHR antagonist methyllycaconitine, indicating that nicotine exerted its specific anti-inflammatory and neuroprotective effects through the α7nAChR.** ***Conclusion*: These findings show that nicotine administration can provide a potential therapeutic pathway for the treatment of peripheral nerve injury, by a direct protective effect through the α7nAChR-mediated cholinergic anti-inflammatory pathway.**

Key words: nicotine, α7nAChR, inflammatory cytokine, peripheral nerve regeneration, crush injury

INTRODUCTION

Peripheral nerve disorders are common neurological problems, which may be caused by traumatic injuries, tumors, and/or iatrogenic lesions. Peripheral nerve injury can result in partial or total loss of motor, sensory, and autonomic function, which could incur substantial financial costs and diminish the quality of life [1]. Compared with the central nervous system, the peripheral nervous system is able to regenerate [2], and the process occurs almost immediately after injury [3]. Over the past century, many therapeutic strategies have been developed for the treatment of peripheral nerve injury, including surgical repair, pharmaceutical intervention, and physical rehabilitation. However, surgical repair does not guarantee full functional recovery to damaged nerves [4]. Current studies have focused on the promising approach involving therapeutic manipulation of the molecular responses to peripheral nerve injury.

The distal segment of the injured nerve undergoes Wallerian degeneration, which is essential for the subsequent processes of regeneration [5]. Therefore, it is important to understand the molecular mechanisms underlying nerve degeneration and regeneration. Within hours, the early inflammatory cytokines in the endoneurium are secreted, mostly by Schwann's cells, and begin to increase in the distal nerve segment [6]. Among these proinflammatory cytokines, tumor necrosis factor alpha (TNF-α) and interleukin (IL-1β), which are up-regulated early and transiently at the site of nerve injury, are considered to play a crucial role in the process of Wallerian degeneration, and are considered initiators of local inflammatory responses [7, 8]. However, overproduction of TNF-α and IL-1β stimulated the production of many other cytokines, and eventually resulted in secondary injury [9]. Research showed that immediate therapy with a TNF-α antagonist enhanced the rate of axonal regeneration after peripheral nerve injury [10]. These data indicated that antagonist treatments may provide new therapeutic options to limit the overabundance of inflammatory cytokines after peripheral nerve injury.

Nicotine is a small organic alkaloid which acts as an agonist on nicotinic acetylcholine receptors (nAChRs) found mainly in the central and peripheral nervous systems and on many other tissues in the body, including immune cells [11, 12]. By attenuating the inflammatory

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response, recent studies showed that nicotine activated the α7nAChR and had a protective role in a variety of tissue damage responses [13-19]. Although the anti-inflammatory effects of nicotine have been demonstrated in different animal models, the protective effects of nicotine during peripheral nerve injury remain controversial. Therefore, the present study was designed to investigate whether nicotine could be beneficial in a rat model with sciatic nerve crush injury.

METHODS

Animal model and drug intervention

Male Wistar rats (weighing 250-300 g) were purchased from Changchun Yisi Experimental Animal Technology Co., Ltd. (Changchun, China). All the animals were cared for and used in accordance with the National Research Council's Guide. The animal protocol was reviewed and approved by the Institutional Animal Care Committee at Harbin Medical University in China.

The model of sciatic nerve crush injury in rats was established as previously reported [20]. Animals were anesthetized by an intramuscular injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and placed prone under sterile conditions. The right sciatic nerve was unilaterally exposed by splitting the gluteal muscle from the femoral greater trochanter to the midthigh. A straight vascular clamp was used to perform a 2-mm long nerve crush injury at the point 5 cm distal to the sciatic notch, for 30 s, and the point was marked by a 6-0 nylon suture tied to the adjacent muscle. Animals were euthanized by carbon dioxide asphyxiation, and sciatic nerves were harvested from the site of injury, to the 15-mm distal point.

Wistar rats (120) were randomly assigned into four groups of 30 rats each, after sciatic nerve crush injury: the saline group (VEH), the nicotine (Santa Cruz Biotechnology, Inc.) treated group (NIC), the methyllycaconitine (MLA; Abcam, Cambridge, MA, USA) treated group (MLA), and the methyllycaconitine + nicotine treated group (M + N). Drugs were injected intraperitoneally, at 1.5 mg/kg for nicotine, at 1, 6, and 12 h after injury, three times a day for seven days. MLA, at 2 mg/kg, was given 30 min prior to administration of nicotine, and saline was injected as a control. The effective doses of drugs were selected based on previous data [16]. In addition, Wistar rats [20] were used for hematoxylin and eosin staining (H&E).

Hematoxylin and eosin staining (H&E)

At 0.25 (6 hours), 1, 3, and 5 days after surgery, the rats were anesthetized and euthanized, and the nerve segments were immediately fixed with 4% paraformaldehyde for 24 h. Paraffin-embedded nerve segments were sectioned longitudinally to a thickness of 4 μm, and stained with H&E stain for morphologic analyses [13].

ELISA assays

The protocol for the ELISA has been previously described [21]. Sciatic nerves were homogenized in ice-cold phosphate-buffered saline (PBS), containing a protease inhibitor cocktail (Roche, Mannheim, Germany). After centrifugation at 10,000 × *g* and 4 °C for 10 min, the supernatant was removed and the pellet was rehomogenized in the same volume of homogenization buffer, with Triton X-100 added to a final concentration of 0.01%. Samples were vortexed and centrifuged, and supernatants were assayed in duplicate, using the TNF-α or IL-1β ELISA kit (Sun Biomedical Technology Co., Ltd, Beijing, China), according to the manufacturers' instructions.

Nerve pinch test

The nerve pinch test was used for evaluating the rate of axonal regeneration, and was usually performed three to seven days after injury, according to the predicted rate of sciatic nerve regrowth [10, 22]. Under light anesthesia, the right sciatic nerve and its tibial nerve branch were exposed. A pair of fine forceps was applied from the distal end of the tibial nerve, and consecutive 1-mm long segments of the nerve were pinched in the proximal direction. Then, the pain reflex, showing contraction of back muscles, was observed, and the distance between the farthest site that produced a reflex response and the original crushed site was recorded as the regeneration distance.

Western blots

Proteins were extracted with lysis buffer added to homogenized sciatic nerve samples, and resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, then transferred to a polyvinylidene difluoride membrane, and blocked with 5% nonfat dry milk. The membrane was then incubated with GAP-43 antibodies (Beijing Bioss Technologies, Inc., Beijing, China), overnight at 4 °C with gentle shaking, and was exposed to anti-mouse immunoglobulin (Ig) G horseradish peroxidase-conjugated antibody for 1 h at room temperature, and finally visualized with enhanced chemiluminescence solution and exposure to hyperfilm.

Morphometric evaluations

Histological assessments were performed as reported previously [23]. By days 7 and 9 after surgery, rats were anesthetized and perfused transcardially with 0.5% glutaraldehyde in 0.1 M phosphate buffer. Short 3 mm segments of the sciatic nerve were then excised, 5 mm under the distal portion of the crush injury. The specimens were fixed in 2.5% phosphate-buffered glutaraldehyde solution for 1 h at room temperature and at 4 °C, until processed. Specimens were then postfixed in 1% OsO4 in PBS, dehydrated in a graded series of alcohol and propylene oxide, embedded in resin, and polymerized at 60 °C. Transverse semithin sections (1 μm) were obtained using an ultramicrotome, and were stained with 1% toluidine blue in 1% sodium tetraborate.

Walking track analyses

Nerve function recovery was assessed by the Sciatic Functional Index (SFI), which indicated the

differences between the injured and the intact contralateral paw. At predetermined times, rats were allowed to walk across a narrow wooden track (7 × 100 cm), darkened at one end. The hind paws of the rats were dipped in ink, resulting in foot prints on the paper. Three measurements were taken from the footprints:
– the print length (PL, distance from the heel to the third toe);
– the toe spread (TS, distance from the first to the fifth toe);
– the intermediate toe spread (ITS, distance from the second to the fourth toe).

All three measurements were taken from both experimental (E) and normal (N) sides, and the three factors that comprised the sciatic function index were calculated as follows:
– print length factor (PLF) = (EPL − NPL)/NPL;
– toe spread factor (TSF) = (ETS − NTS)/NTS;
– intermediary toe spread factor (ITF) = (EIT − NIT)/NIT [24].

The SFI was calculated using these data, according to the following equation: SFI = −38.3 × PLF + 109.5 × TSF + 13.3 × ITF −8.8.

Statistical analysis

SPSS 19.0 software (SPSS, Chicago, IL, USA) was utilized to analyze the data, which were expressed as mean ± SEM. All statistical analyses were assessed using the one-way analysis of variance test followed by Tukey's test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

General observations of the rat model with sciatic nerve crush injury

When the crush injury of rat sciatic nerve was just finished, complete crush of the sciatic nerves was confirmed through the following observations: presence of a translucent band across the nerve, right limb dysfunction, and presence of extensive Wallerian degeneration assessed by H&E staining. In contrast, well-arranged and distributed nerve fibers were observed in normal specimens. By day 1 after injury, swelling of axons, myelin abnormalities, endoneurial edema, and hypertrophy of Schwann's cell cytoplasm were observed. By day 3, irregularities in axon morphology, the collapse of myelin, endoneurial edema, and a greater number of Schwann's cells were present. By day 5, we found the axons had collapsed and more Schwann's cells were detected (*figure 1*).

Nicotine attenuated the expression of TNF-α and IL-1β after sciatic nerve crush injury

Nicotine has been identified as a potent suppressor of proinflammatory cytokine production in lung and liver injury [17, 18]. In our previous study, protein expression of TNF-α was increased in early stages of sciatic nerve crush injury [25]. To investigate the effects of nicotine on cytokine production, protein expressions of TNF-α and IL-1β were measured by ELISA on day 1 after crush injury. The results demonstrated that the production of TNF-α was significantly lower in the NIC group (55.7 ± 6.6 pg/mg) compared to both the VEH (95.6 ± 5.6 pg/mg) and M + N (88.9 ± 7.1 pg/mg) groups,

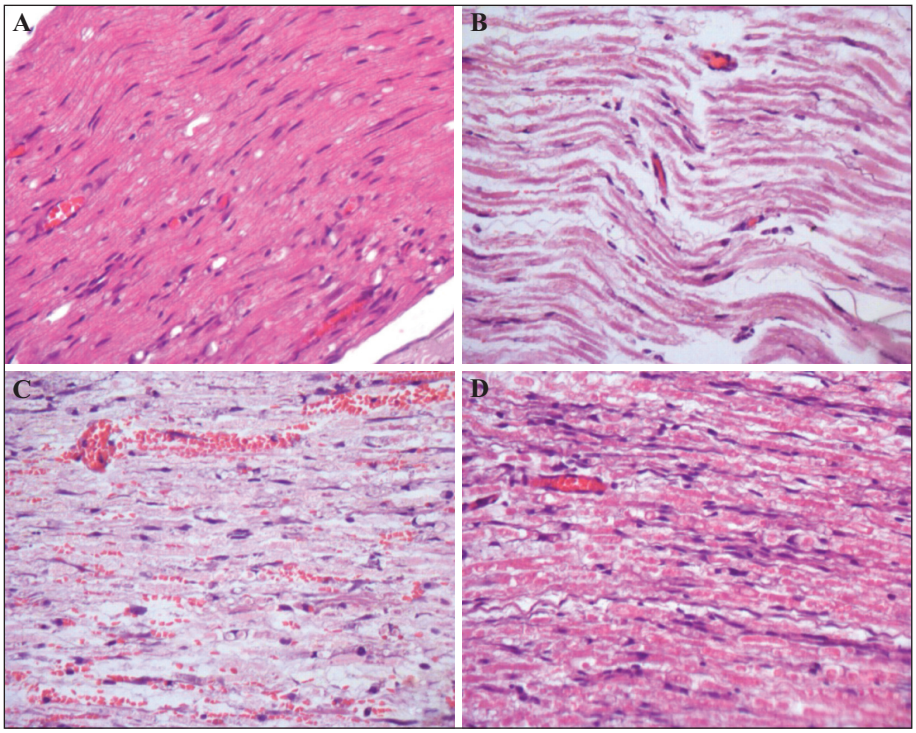

Figure 1
Longitudinal sections with H&E staining (×400). Sections of the sciatic nerve distal to the crush site showed extensive Wallerian degeneration in injured specimens. **A)** normal specimens; **B)** day 1 after crush injury; **C)** day 3 after crush injury; **D)** day 5 after crush injury. n = 5/group.

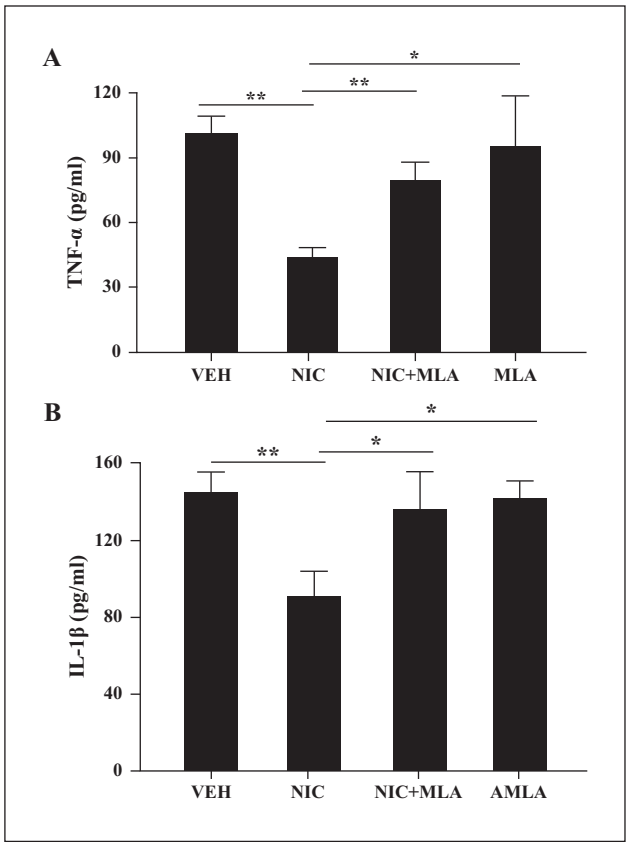

Figure 2
Effect of nicotine on local TNF-α and IL-1β measured by ELISA on day 1 after sciatic nerve crush injury. Data are expressed as a mean and SEM. ** $P < 0.01$ *versus* NIH, $n = 8$/group.

while there was no significant difference between the VEH and MLA groups. The IL-1β level was significantly higher in both the VEH (146.7 ± 8.4 pg/mg) and the M + N (137.6 ± 6.1 pg/mg) groups compared to the NIC (89.3 ± 5.5 pg/mg) group. In contrast, there was no significant difference between the VEH and MLA groups. Nicotine significantly decreased local TNF-α and IL-1β levels, and co-administration of MLA reversed this effect (*figure 2*).

Nicotine improved the rate of sensory nerve regeneration after crush injury

We performed the pinch test to measure growth of sensory axons. McQuarrie *et al*. [22] showed that the anticipated regeneration distance, on day 5 after crush, equaled 13.6 mm, which was close to our finding in the VEH group (13.7 ± 0.5 mm), the M + N group (14.4 ± 0.9 mm), and the MLA group (14.0 ± 0.5 mm). In addition, the regeneration distance on day 5 after crush in the NIC group was significantly longer than that in the other groups, equaling 19.1 ± 0.7 mm (*figure 3*).

Nicotine increased the expression level of GAP-43 after sciatic nerve crush injury

Then we detected the expression of GAP-43, which was induced in regenerating sciatic nerve fibers and was regarded as a marker for axonal regeneration [10, 26]. Five days after crush injury, a significant increase in

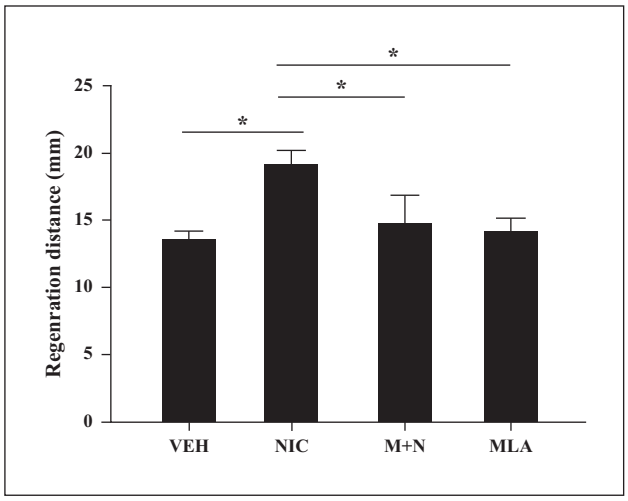

Figure 3
Effect of nicotine on nerve regeneration distance measured by pinch test on day 5 after sciatic nerve crush injury. Data are expressed as a mean and SEM. * $P < 0.05$ *versus* NIH, $n = 8$/group.

GAP-43 expression was found in the NIC group compared with the VEH, M + N, and MLA groups (*figure 4*). This effect of nicotine on axonal regeneration was abolished by MLA, indicating that nicotine acted through α7nAChR.

Nicotine promoted sciatic nerve morphological recovery after crush injury

Morphometric analysis was commonly used to evaluate recovery from nerve injuries [27], and our study showed that nicotine was capable of enhancing nerve

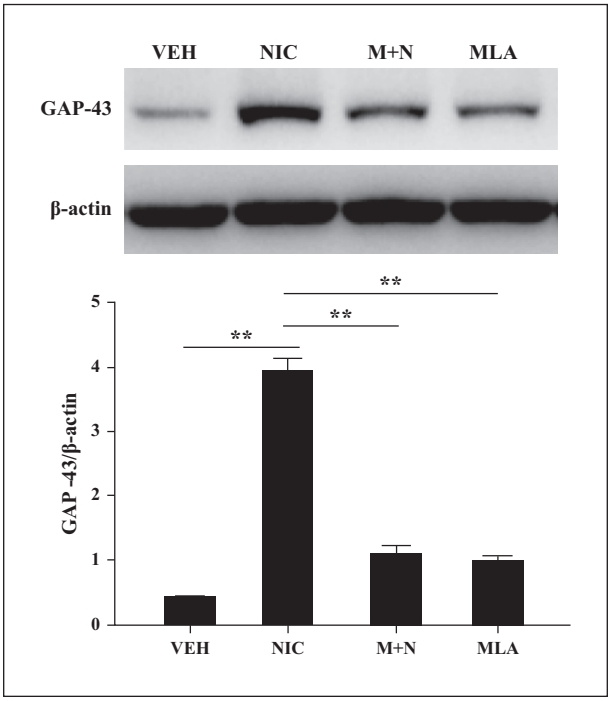

Figure 4
Effect of nicotine on expression level of GAP-43 by western blot on day 5 after sciatic nerve crush injury (top). The relative protein level of GAP-43 to β-actin was determined using the ImageJ program (bottom). ** $P < 0.01$ *versus* NIH.

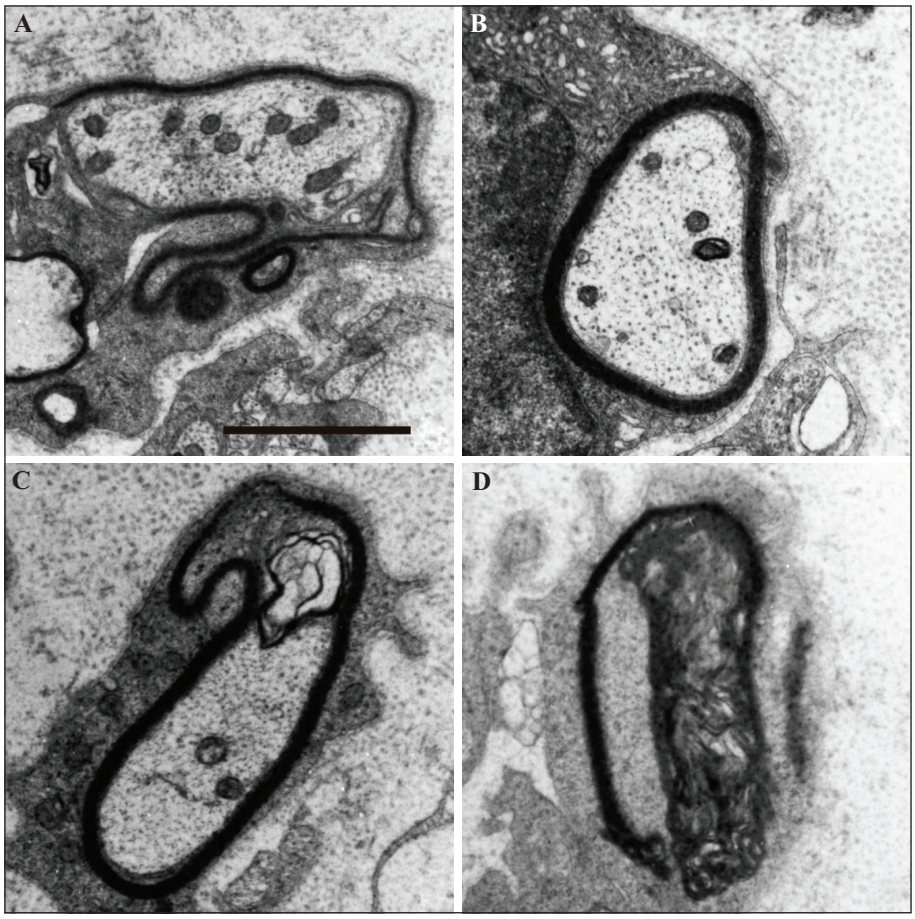

Figure 5
Transmission electron micrographs showing the ultrastructure of sciatic nerve fiber (×20,000) day 9 after sciatic nerve crush injury. **A)** the VEH group; **B)** the NIC group; **C)** the M + N group; **D)** the MLA group. Scale bar = 2 μm.

regeneration after injury. We found that sections of the sciatic nerve distal to the crush site were removed for morphological observation at seven and nine days after injury. Myelinated nerve fibers from regeneration were seldom observed on day 7 after crush injury, while they could be seen on day 9. Most myelin sheaths of myelinated fibers in the NIC group were well developed and intact, and most of them in the other groups were characterized by poor development and incomplete structures (*figure 5*).

Nicotine enhanced the recovery of motor function after crush injury

Walking track analysis illustrated the recovery of sciatic nerve function, and the improvement in SFI values indicated that some regenerated axons had reached the target organ. No differences in SFI values were observed among all groups on postoperative day 7. However, the SFIs in the NIC group were significantly higher than those observed in the VEH and

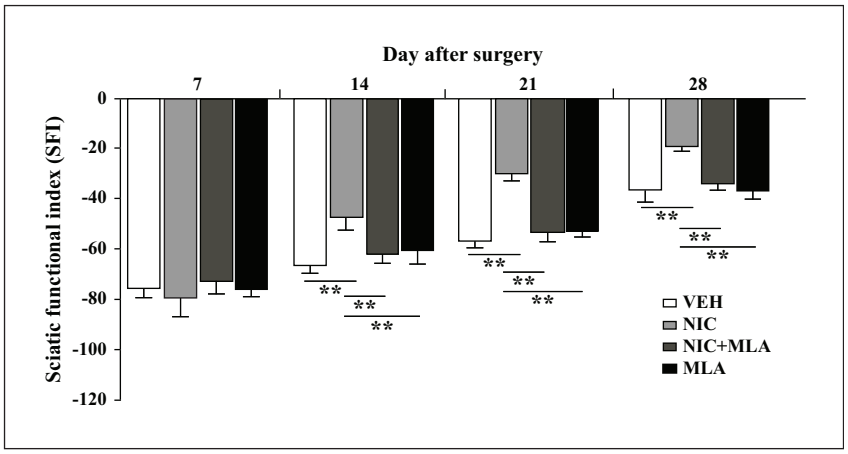

Figure 6
Comparison of functional recovery determined by the Sciatic Functional Index (SFI) on days 7, 14, 21, and 28 after sciatic nerve crush injury. Data are expressed as a mean and SEM. ** $P < 0.01$ *versus* NIH, $n = 8$/group.

MLA groups on postoperative days 14, 21, and 28. The improvement in SFI values in the M + N group was not significant when compared to the NIC group, indicating that MLA partly reversed the effects of nicotine. The results confirmed that nicotine enhanced the recovery of motor function after crush injury (*figure 6*).

DISCUSSION

Peripheral nerves are often damaged by penetrating injury, crush, stretch, and ischemia, and the incidence was estimated to affect at least 500,000 new patients annually in the USA [23]. Injured peripheral nerves first show subcellular and molecular effects in the involved sensory and motor neurons, and are known to have a considerable capacity for regeneration. However, recovery from these injuries is rarely complete. As a consequence, the patients experience persistent impairment, chronic pain, motor dysfunction, and inappropriate autonomic responses [1].

Axonotmesis is commonly seen in crush injury, causing severe sensorimotor impairments and functional disabilities [28]. Crush injury is therefore an appropriate model to study the cellular and molecular mechanisms of peripheral nerve regeneration, and to observe the role of different factors in the nerve regeneration process. It is also an appropriate model to investigate the effect of various pharmacological treatments on experimental regeneration [8, 10, 24, 27].

The present study was designed to confirm the hypothesis that nicotine enhanced nerve regeneration by attenuating proinflammatory responses in rat models of sciatic nerve crush injury. The nerve crush injury in our model was assessed by observations during the operation, by H&E staining, and by the disability of limb functions after the operation.

After crush injury of peripheral nerves, Wallerian degeneration occurred in the distal segment, which was directly regulated by several inflammatory cytokines and was also indispensable for the subsequent processes of nerve regeneration. Of these proinflammatory cytokines, TNF-α and IL-1β are considered to play a crucial role in the process of Wallerian degeneration. As an initiator of local inflammatory responses, they are up-regulated early, and transiently, at the site of nerve injury. Peripheral nerves have a considerable capacity for regeneration after injury, and the process is regulated by inflammatory cytokines expressed in the injured nerves at an early phase of injury [10]. It is therefore important to understand the function of inflammatory cytokines and cytokine-regulatory proteins after peripheral nerve injury.

Nicotine has been shown to have anti-inflammatory effects through the activation of α7nAChR, expressed mainly in neurons, astrocytes, epithelial cells, adipocytes, fibroblasts, keratinocytes, and immune cells [29-32]. It has also been demonstrated that the activation of the anti-inflammatory cholinergic pathway, by nicotine administration, had anti-inflammatory effects in many inflammatory disease states.

In a model for acute respiratory distress syndrome (ARDS), nicotine was shown to be very effective at decreasing the levels of TNF-α, IL-1, IL-6, and lung edema. Nicotine has been shown to decrease inflammatory cytokine production by macrophages and other mononuclear cells, which may account for the protective effect found in ARDS [13]. In the renal ischemia/reperfusion model, nicotine pretreatment protected renal function by decreasing TNF-α, creatine kinase (CK), and high mobility group box protein-1 (HMGB1) levels, and by regulating the neutrophil infiltrate in an α7nAChR-dependent manner, 24 h after reperfusion [14]. In the transient forebrain ischemia model, the neuroprotective effects of nicotine inhibited microglial proliferation, and overexpression of tumor necrosis factor alpha (TNF-α), and IL-1β, suggesting a potential therapeutic effect against neural damage induced by brain inflammation following ischemia/reperfusion [15]. In the spinal cord injury model, injection of nicotine, 2 h after the trauma, showed neuroprotective effects by attenuating the induction of oxidative stress, activation of DNA-binding activities of redox-responsive transcription factors, and expression of monocyte chemoattractant protein-1 (MCP-1) and TNF-α. Treatment of mice with nicotine significantly reduced plasma levels of TNF-α protein after 3, 6, and 24 h of reperfusion, and plasma levels of IL-6 protein after 3 and 6 h of reperfusion. However, increased levels of TNF-α and IL-6 mRNA were not significantly altered, indicating that the inhibitory effect was through a posttranscriptional mechanism [17]. In general, previous reports suggested that nicotine could be a promising neuroprotective agent in treatment of inflammatory diseases [13-19].

The present study reported for the first time that nicotine had a strong anti-inflammatory role through α7nAchR, and further provided significant neuroprotection against peripheral nerve injury induced by crush. Our previous study showed that localized expressions of α7nAChR and TNF-α were increased during the early stages of sciatic nerve injury, indicating that α7nAChR and TNF-α could play a role in the process of regeneration after peripheral nerve injury [25]. In this study, we showed that administration of nicotine significantly attenuated local expressions of TNF-α and IL-1β at 1 day after crush injury, and MLA reversed the protective effects of nicotine on crush-induced overexpression of both the TNF-a and IL-1β levels, indicating that nicotine attenuated the inflammatory response by α7nAChR.

In addition, our results showed that, during the study period nicotine treatment led to better nerve regeneration and faster functional recovery after crush injury. The pinch test was used to measure the influence of nicotine on peripheral nerve regeneration. The regeneration after vehicle treatment (VEH; mean of 13.7 mm) was similar to the previously reported values (13.6 mm) at five days after the injury for rat crushed sciatic nerves [22]. Nicotine administration significantly improved the regeneration (mean of 19.1 mm) compared with the vehicle-treated animals. However, regeneration in the M + N and MLA groups was not significantly different from that in the VEH group. GAP-43, a marker for axonal regeneration, is produced in neuronal soma and transported axonally into the growth cone of regenerating axons [2].

Nicotine therapy increased localized GAP-43 levels, and MLA attenuated the promoting effect. Morphometric analysis also showed that nicotine enhanced regeneration of myelinated fibers, with intact myelin sheaths observed on day 9 after crush injury. SFI is a standard measurement for evaluating crush peripheral nerve injury, and is used to evaluate the degree of functional recovery. Our study showed that the SFI values of the NIC group were significantly better than those of the VEH group on days 14, 21, and 28 after surgery. Nicotine enhanced the recovery of motor function after crush injury, and MLA partly reversed the role of nicotine. All these data indicated that nicotine promoted nerve regeneration and functional recovery through the activation of α7nAChR.

CONCLUSION

Our data demonstrate that nicotine administration inhibits overexpression of proinflammatory cytokines (TNF-α and IL-1β) and promotes nerve regeneration and functional recovery after sciatic nerve crush injury through activation of α7nAChR-mediated cholinergic anti-inflammatory pathways. These findings may have significant implications for the comprehension of the cellular and molecular mechanisms involved in nicotine-induced neuroprotection, and suggest that agonists of nAChRs could constitute attractive therapeutic drugs for the treatment of peripheral nerve injury.

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