Journal of Traditional and Complementary Medicine 14 (2024) 276–286

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: www.elsevier.com/locate/jtcme

Rosmarinic acid Ameliorates neuronal regeneration in the bridging silicone rubber conduits of the sciatic nerve in taxol-treated rats☆

Ping-Ling Chiu [a,1], Mei-Chen Lin [a,1], Shih-Tien Hsu [a,b,c,1], Tin-Yun Ho [a], Yung-Hsiang Chen [a,d], Chung-Chia Chen [e,f,**], Yueh-Sheng Chen [a,*]

[a] Program for Aging, Department of Biomedical Engineering, College of Biomedical Engineering, School of Medicine, Graduate Institute of Chinese Medicine, Graduate Institute of Integrated Medicine, School of Chinese Medicine, China Medical University, Taichung, 404, Taiwan
[b] Department of Obstetrics and Gynecology and Women's Health, Taichung Veterans General Hospital, Taichung, 407, Taiwan
[c] Center for General Education, Ling Tung University, Taichung, 408, Taiwan
[d] Department of Psychology, College of Medical and Health Science, Asia University, Taichung, 413, Taiwan
[e] Linsen Chinese Medicine and Kunming Branch, Taipei City Hospital, Taipei, 103, Taiwan
[f] School of Chinese Medicine for Post Baccalaureate, I-Shou University, Kaohsiung City, 840, Taiwan

ARTICLE INFO

Keywords:
Inflammation
Neuropathy
Peripheral nerve injury
Regeneration
Rosmarinic acid
Silicone rubber conduit
Taxol

ABSTRACT

Background and aim: Taxol modulates local inflammatory conditions in peripheral nerves, which may impair their regeneration and recovery when injured. This study aimed to determine the effects of rosmarinic acid (RA, a polyphenol constituent of many culinary herbs) on the regeneration of the sciatic nerves in the bridging conduits.
Experimental procedure: In the cell study, RA decreased nuclear factor (NF)-κB activity induced by taxol in a dose dependency. In the animal model, taxol-treated rats were divided into 3 groups (n = 10/group): taxol (2 mg/kg body weight for 4 times) and taxol + RA (3 times/week for 4 weeks at 20 and 40 mg/kg body weight) groups. Macrophage infiltration, calcitonin gene-related peptide (CGRP) expression levels, neuronal connectivity, animal behavior, and neuronal electrophysiology were evaluated.
Results and conclusion: At the end of 4 weeks, macrophage density, CGRP expression level, and axon number significantly increased in the RA group compared with the taxol group. The RA administration unaffected heat, cold plate licking latencies, and motor coordination. Moreover, the 40 mg/kg RA group had significantly larger nerve conduction velocity and less latency compared to the taxol group. This study suggested that RA could ameliorate local inflammatory conditions to augment the recovery of regenerating nerves by accelerating their regrowth and improving electrophysiological function in taxol-treated peripheral nerve injury repaired with the silicone rubber conduit.

1. Introduction

Peripheral nerve injury (PNI) may cause interrupted axon continuity, distal neuropathy, nerve fiber degeneration, and eventually neuronal death.[1,2] Processes occurring after PNI frequently bring permanent damage to sensory and motor functions as well as neuropathic pain and other secondary problems. In addition, peripheral nervous system damage may impose severe loss of autonomic function and reduced quality of life and have significant social consequences in terms of health care and long-term illness.[3–5]

Herein, the pathogenesis of PNI can be divided into primary and secondary injuries. Primary injuries refer to mechanical damage caused by trauma, including irreversible damage to the vascular structure, axonal rupture, and nerve cell death. Meanwhile, secondary injuries are generally classified as chemical irritation damage, including ischemic damage, glutamate toxicity damage, peroxide damage, excessive intracellular calcium accumulation damage, and neuronal apoptosis or

☆ **Footnotes:** Rosmarinic Acid Ameliorates Neuronal Regeneration.Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

* Corresponding author.
** Corresponding author. Linsen Chinese Medicine and Kunming Branch, Taipei City Hospital, Taipei, 103, Taiwan.
E-mail addresses: r3824222@yahoo.com.tw (P.-L. Chiu), lmc9363@mail.cmu.edu.tw (M.-C. Lin), sthsu@vghtc.gov.tw (S.-T. Hsu), tyh@mail.cmu.edu.tw (T.-Y. Ho), yhchen@mail.cmu.edu.tw (Y.-H. Chen), dal06@tpech.gov.tw (C.-C. Chen), yuehsc@mail.cmu.edu.tw (Y.-S. Chen).
[1] These authors contributed to the work equally and should be regarded as co-first authors.

https://doi.org/10.1016/j.jtcme.2024.03.004
Received 7 July 2023; Received in revised form 3 January 2024; Accepted 5 March 2024
Available online 5 March 2024

List of abbreviations

Bcl-2	B-cell lymphoma 2
CCK-8	colorimetric cell counting kit-8
CGRP	calcitonin gene-related peptide
DRGs	dorsal root ganglias
FG	fluorogold
IHC	Immunohistochemical
IP	intraperitoneally
JNK	N-terminal c-Jun protein kinase
NADPH	nicotinamide adenine dinucleotide phosphate
NF	nuclear factor
PNI	peripheral nerve injury
RA	rosmarinic acid
RLUs	relative luciferase units
ROS	reactive oxygen species

fibrotic degeneration at the adjacent damaged site. In particular, the secondary injury has mainly a foundation in the chronic inflammatory response.[6]

Given that taxol (paclitaxel) was first isolated from Pacific Yew in 1971, it has been widely used in various cancer treatments, inclusive of lung cancer, breast cancer, ovarian cancer, and cervical cancer. The mechanism of taxol is to interfere with the function of microtubules during cell division and leads the proliferating cancer cell death. In addition, taxol has dramatically different functions on neuronal cells and non-neuronal cells. Moreover, taxol activates N-terminal c-Jun protein kinase (JNK) and phosphorylation of B-cell lymphoma 2 (Bcl-2) in cancer cells, thereby causing cell apoptosis. In contrast to the neuronal cell, it has been presented that taxol prompts cell damage via the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase for inciting oxidative cell apoptosis. NADPH oxidase is a cytoplasmic enzyme that produces reactive oxygen species (ROS) by electron transfer. Furthermore, ROS overproduction expressed by NADPH oxidase is a key to triggering neurodegenerative diseases, and it is also related to the side effects of chemotherapy. The side effect of nerve damage after taxol treatment to the cancer patient has not followed apoptosis through BCL-2 phosphorylation but is caused by NADPH-mediated oxidative damage.[7–11]

Meanwhile, anti-inflammatory and antioxidant activities are correlated with neuroprotection. Among the potential drug candidates, rosmarinic acid (RA) has attracted a lot of attention. RA purified from rosemary is a water-soluble polyphenolic compound, which resists ROS damage resulting from photosynthesis. As a natural antioxidant, RA has anti-inflammatory, anti-apoptotic, anti-tumor, and neuroprotective properties. Herein, in the rat neuropathic pain model, RA reveals a significant protective ability. Moreover, it has represented neuroprotective effects for related neurological diseases, such as Parkinson's disease, Alzheimer's disease, and even stroke.[12–16] Therefore, RA has therapeutic potential against taxol-mediated nerve damage.

In previous studies, our team successfully established an animal model of taxol-treated peripheral nerve regeneration in rats. The results illustrated that taxol exposure increased systemic inflammation and restrained peripheral nerve regeneration. In addition, our findings demonstrated that electroacupuncture facilitated peripheral nerve regeneration in taxol-treated rat models because electroacupuncture can drive muscle contraction to accelerate blood flow. Thus, this strategy improves peripheral perfusion by supporting more nutrients to nerves, for the reason that obtains better nerve regeneration and reconstruction.[17,18]

In this study, RA was applied to *in vitro* anti-inflammatory studies and *in vivo* peripheral nerve regeneration on taxol-treated rat models based on our previous achievements. Silicone is a material with good biocompatibility and is widely used in various medical devices. Since the diffusion rate of substances in silicone is very stable, it is used for drug-carrying treatment. It will be a new research direction to combine silicone with RA for nerve regeneration. In brief, this study aims to evaluate the effects of different RA concentrations assisting peripheral nerve regeneration in taxol-treated rat models with a 10-mm sciatic nerve gap in silicone rubber conduits. After a four-week recovery period, the experimental results of macrophage infiltration in regenerated nerves, retrograde fluorescent gold-labeled dorsal root ganglias (DRGs), electrophysiological stimulation, animal behavior patterns, and tissue sections were collected. The above experiments can prove whether or not RA can promote nerve regeneration in taxol-treated rat models.

2. Materials and methods

2.1. Materials

Silicone rubber tubes (used as a physical guide for regenerating axons, providing a protected environment for nerve regeneration) were obtained from Helix Medical, Inc. (Carpinteria, CA, USA) with 1.47 and 1.96 mm in inner and outer diameters, respectively. RA was acquired from Sigma-Aldrich (536,954; St. Louis, MO, USA). Cremophor EL (a formulation vehicle used to improve the solubility of various poorly water soluble drugs, including the anticancer agent taxol) and dimethyl sulfoxide (DMSO) solution was acquired from Sigma-Aldrich (St. Louis, MO, USA). Meanwhile, the fluorogold (FG) solution was from Fluorochrome (Denver, CO, USA). Other chemicals and reagents used were analytical grade. Taxol Formoxol injection was purchased from Yung Shin Pharmaceutical Industrial Co., LTD., Taiwan. Colorimetric cell counting kit-8 (CCK-8) was purchased from MedChemExpress, USA. D-Luciferin was purchased from Xenogen (Hopkinton, MA). We have conducted biocompatibility studies on silicone and the use of the excipient Cremophor EL-RA in the pilot study. The results showed no significant cytotoxicity or adverse reactions.

2.2. Cell culture

Cell culture was assessed by recombinant HepG2/nuclear factor (NF)-κB cells, which carried NF-κB-driven luciferase genes (Cellomics Technology, Halethorpe, MD). HepG2/NF-κB cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) and incubated at 37 °C with 5% CO_2.

HepG2/NF-κB cells (1×10^5 cells) were seeded in a 96-well plate and incubated at 37 °C overnight. Taxol (100 ng/mL), or various amounts of RA were then added to the cells and incubated at 37 °C for 24 h. Cell viability was analyzed by CCK-8 assay. Luciferase assay was performed as described previously. Relative NF-κB activity was calculated by dividing the relative luciferase units (RLUs) of compound-treated cells by the RLUs of solvent-treated cells.[19]

2.3. Animal study

Female adult Sprague-Dawley rats (LASCO, Taipei, Taiwan, ROC) were used in the study. Moreover, animal studies were performed in line with the "Guide for the Care and Use of Laboratory Animals", and the protocols were approved by the Ethics Committee of the China Medical University, Taiwan (Project identification code: CMUIACUC-2020-177). The PNI animal model was designed by taxol treatment and surgical truncation. Then, the therapeutic effect of RA on PNI in rats was evaluated. Subsequently, after being adaptively fed for 7 successive days, the rats were intraperitoneally (IP) injected with taxol (2 mg/kg body weight) that dissolved in Cremophor EL solution 4 times a week. The dose used in this study was based on our previous studies, where we established the rat model of taxol-induced peripheral nerve injury and evaluated the effects of electroacupuncture and other interventions on

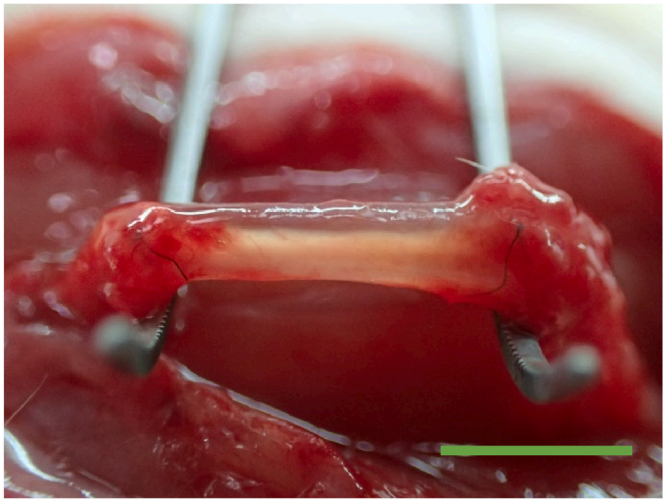

Fig. 1. Representative image depicting the implanted silicon conduit supporting the successfully regenerated nerve cable in rats. Scale bar = 5 mm.

nerve regeneration. This dose was chosen to induce significant neuropathic pain and nerve degeneration without causing severe systemic toxicity or mortality in rats.[20,21] After the taxol treatment, the right sciatic nerve of the rat was surgically divided into proximal and distal segments. Both stumps were fixed with a suture through the epineurium and a silicone rubber tube with a 10-mm gap apart. All taxol-treated rats were randomly allocated into three groups (10 for each group), groups received RA administration (IP, 3 times/week for 4 weeks) at 0, 20, and 40 mg (in 5% DMSO)/kg (body weight) groups, respectively. After four weeks of nerve repair, relevant behavior and functional tests was performed. All rats would be sacrificed for histological analysis after five weeks.[17,18]

2.4. Hargreaves's test for thermal hyperalgesia

Thermal hyperalgesia was assessed by measuring the animal behavior using Hargreaves' test IIT Canalgesiometer (IITC Life Sciences, SERIES8, Model 390 G). Rats were housed on a hot/cold plate (Panlab, Harvard Apparatus, Holliston, MA, USA) and measured licking latency for 5 min. The temperature of the heat stimulation was controlled at 40 °C and the cold plat apparatus at 4 °C.[17,18]

2.5. Motor coordination test

Motor coordination has been used to evaluate the state of nerve recovery in rats. The taxol-treated rats were placed on a rotating rod with an initial rotating speed of 4 rpm and accelerated by 1.8 rpm per 8 s. The motor performance of taxol-treated rats was measured from the duration that the rats stayed on the rotating rod. In addition, an accelerating Rotamex Columbus instrument (Rotamex rotarod, Columbus Instruments, Columbus, OH) was used in this test.[17,18]

2.6. Electrophysiological test

Electrophysiological testing was performed using needle electrodes to stimulate gastrocnemius muscle and the nerve conduction velocity (NCV), amplitude, latency, and evoked muscle action potentials (MAPs) were recorded through BIOPAC Systems, Inc. (Goleta, CA, USA).[17,18]

2.7. FG tracer labeling

The deeply anesthetized rats have injected with FG solution using Hamilton micro-syringe into the common peroneal and posterior tibial nerves. The injection site needs to be kept for 1 min to prevent backflow of the tracer. After 5 days, the animals were sacrificed and perfused with saline and paraformaldehyde. Then, after perfusion, the ipsilateral L4 and L5 DRGs were taken out for preservation. After frozen sectioning, the density of labeled neurons was quantified by a fluorescence microscope (Olympus ckx41, Center Valley, PA, USA).[17,18]

2.8. Histological analyses

After 4 weeks of recovery from the silicone rubber catheter, the regenerated nerves were removed and the middle areas of the nerves were dissected (Fig. 1). The nerve segments were placed in a 2% glutaraldehyde solution for 24 h of fixation. The thin nerve sections (1 μm) were stained with toluidine blue and examined under light microscopy. On the one hand, the integrity of nerve regeneration from tissue sections was evaluated. On the other hand, immunostaining was used to label

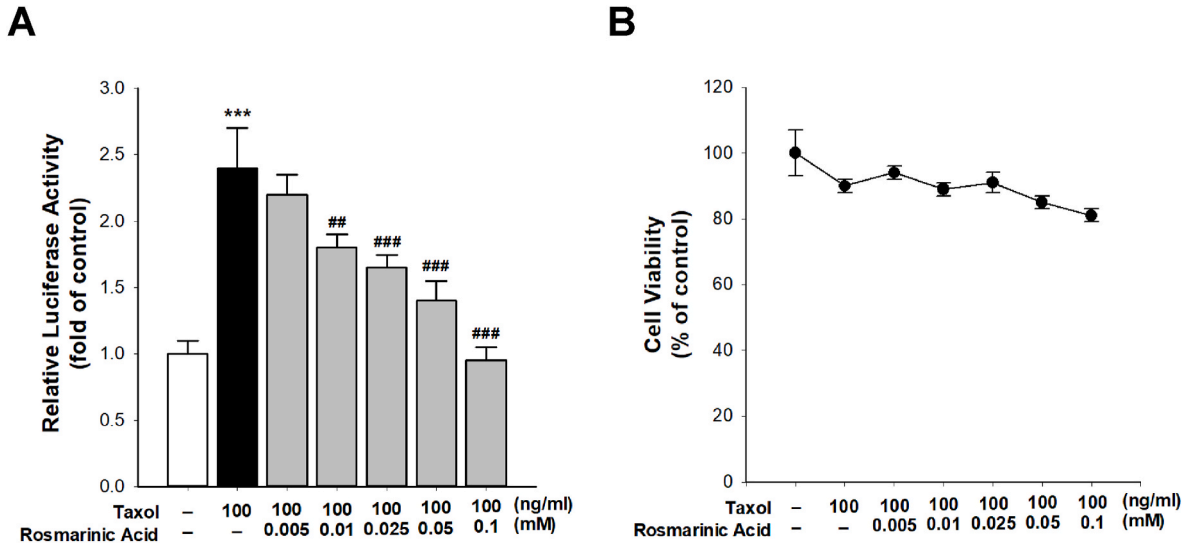

Fig. 2. RA suppressed taxol-induced NF-κB activation in cells. HepG2/NF-κB cells were treated with 100 ng/mL taxol and various amounts of RA. (A) NF-κB activity and (B) cell viability were measured 24 h later by luciferase and CCK-8 analyses. Results are expressed as relative NF-κB activity (which is presented as the comparison with RLU relative to solvent-treated cells) and viability, respectively. Values are mean ± SEM (n = 6). $^{***}P < 0.001$, compared with mock control. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with taxol group.

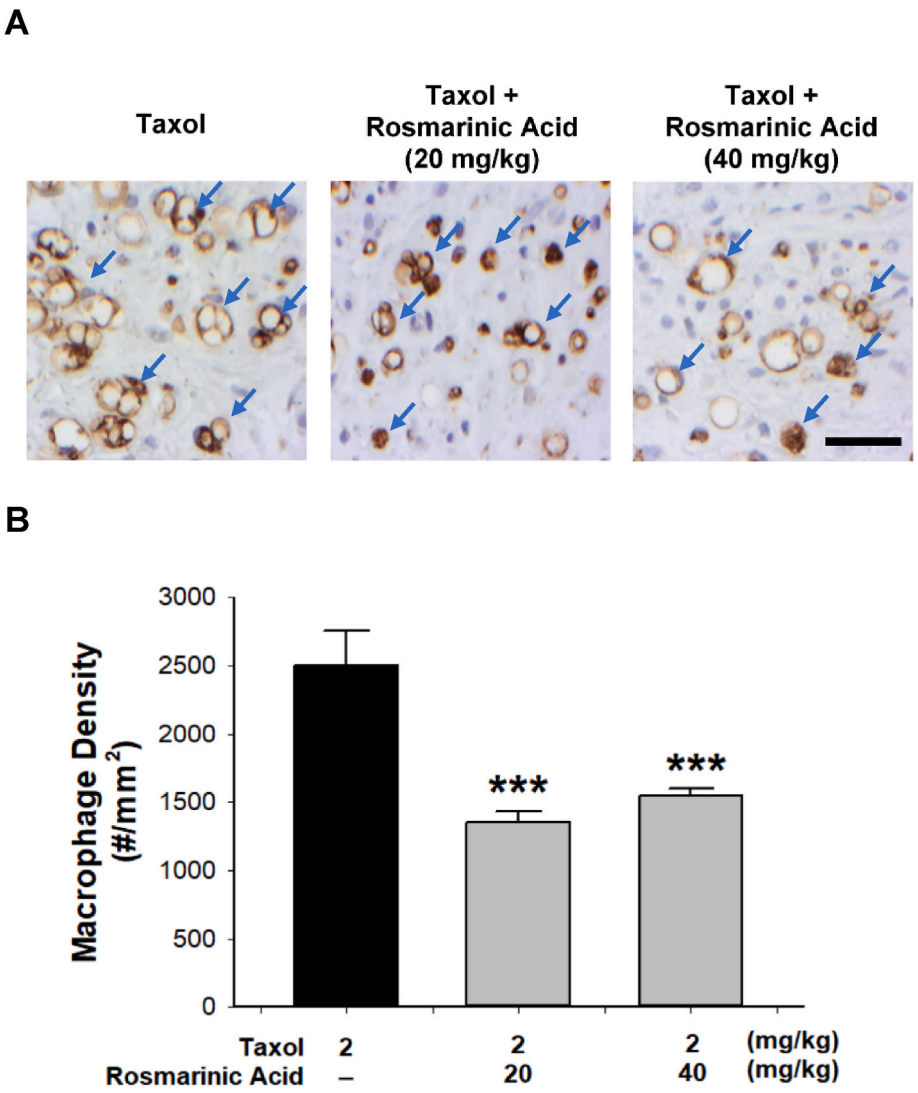

Fig. 3. Effects of RA on macrophage infiltration in taxol-treated rats. (A) Representative images of macrophages (arrows) and (B) quantitation of macrophage infiltration. Values are mean ± SEM (n = 10). ****$P < 0.001$ compared with taxol group. Scale bar = 50 μm.

CD68 (a marker for the various cells of the macrophage lineage) in the distal region of the regenerated nerve. After the primary [anti-calcitonin gene-related peptide (CGRP) antibody 1:1000, Merck, USA] and secondary (Novolink Polymer RE7112) antibodies were processed,[22,23] the image analysis software (Image-Pro Lite, Media Cybernetics, Rockville, MD, USA) was used to determine the ratio of macrophage infiltration. Images were then acquired with ZEISS Axioskop (Germany).[17,18]

2.9. Statistical analyses

Data were collected by the same observer and expressed as mean ± standard error of the mean (SEM), and comparisons between groups were made by one-way analysis of variance using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The Tukey test was then used as a *post hoc* test for multiple comparison. Statistical significance was set at $P < 0.05$.

3. Results

3.1. In vitro NF-κB activation

In the *in vitro* test, the inhibitory effect on the inflammatory response was evaluated and the growth of the cells was examined. Fig. 2 shows the results of luciferase and cell viability assay. As shown in Fig. 2A, taxol increased the NF-κB activity by 2.39 ± 0.28 fold, compared with untreated cells. RA decreased NF-κB activity induced by taxol, and the decrease displayed a dose dependency. The maximal inhibition (59.59% ± 4.05%) was observed at 0.1 mM RA. No clearly high cytotoxic effects were observed, judged by CCK-8 assay. These findings suggested that RA significantly suppressed NF-κB activity induced by taxol in cells. Therefore, in all groups, the cell viability was consistently higher than 80% (Fig. 2B), showing the good biological properties of the material.

3.2. Macrophage infiltration

Macrophages are well studied at sites of nerve injury and are critical for nerve regeneration, Fig. 3 shows the test results of macrophage density at the injured nerve after taxol treatment, meanwhile, Fig. 3A is the photo of the taxol group and the taxol plus RA content of 20 and 40 mg/kg, respectively, and the calculated results of the density are shown in Fig. 3B. The study evaluated the physiological state of regenerated nerves through the content and density of macrophages, which can identify pathogens and regulate the inflammatory response. Based on the results, the macrophage density in the taxol group was higher, and the cells aggregated with each other, thereby indicating that the inflammatory response to the wound was stronger. With the addition of RA, the figures show a smaller number of macrophages with lower

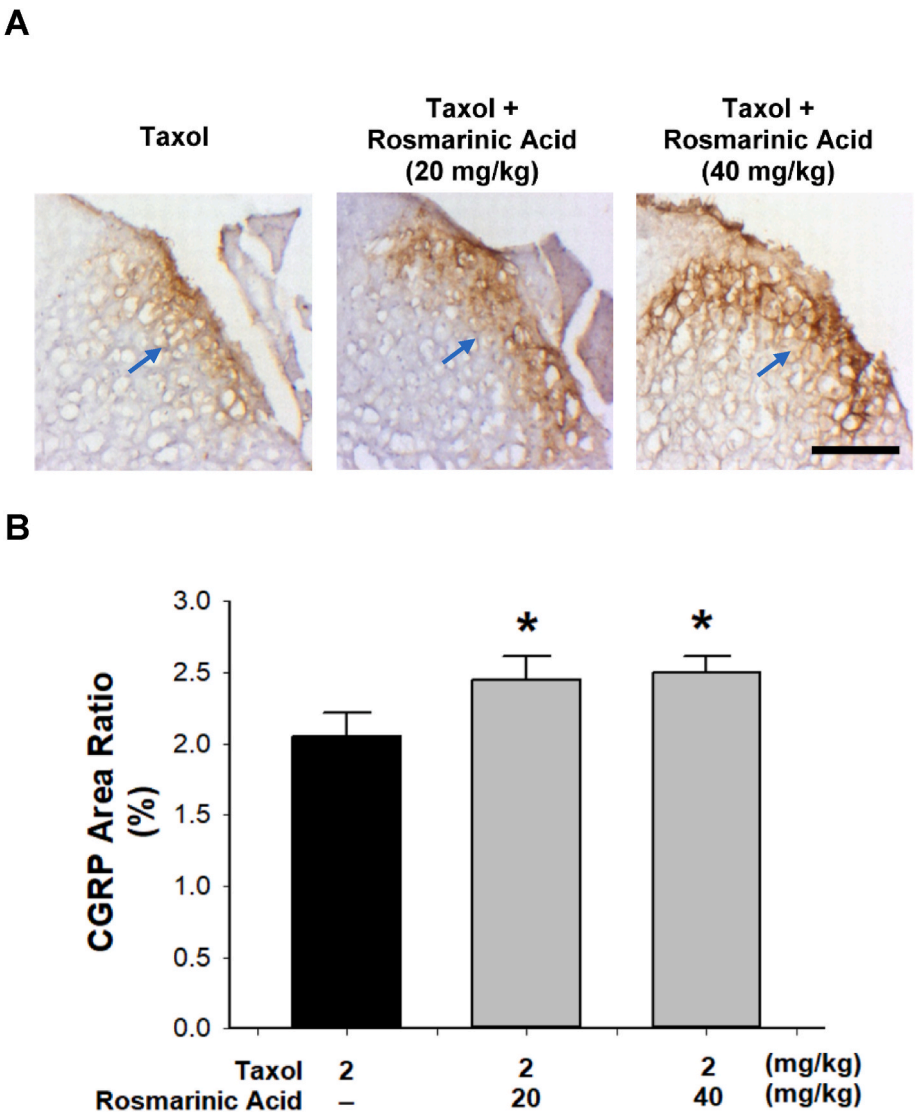

Fig. 4. Effects of RA on CGRP expression in taxol-treated rats. (A) Representative images of CGRP-labeled fibers (arrows) and (B) comparison of the ratio of CGRP expression area. Values are mean ± SEM (n = 10). *$P < 0.05$ compared with taxol group. Scale bar = 100 μm.

density, thus showing an increase in the phagocytic capacity of macrophages and a slowing of the inflammatory response in the wound.

3.3. CGRP expression

Immunohistochemical (IHC) staining demonstrated the presence of CGRP-labeled fibers in the area of the lamina of the dorsal horn in all rats. The expression of CGRP was also used to assess the effect on nerve and activated macrophage production after injury. The result of IHC staining showing the area occupied by CGRP immunoreactive cells is shown in Fig. 4A. Increased levels of RA (20 and 40 mg/kg) significantly increased the release of CGRP (Fig. 4B).

3.4. FG tracer labeling

The fluorescent gold number measurement results were shown in Fig. 5A. The group added to RA (40 mg/kg) significantly increased the number of cells stained compared to the taxol group (Fig. 5B). The research used inverse tracing to evaluate the conduction capacity of regenerated nerves and verified by fluorescent dyes. The nerve fibers in the RA-added group recovered better.

3.5. Axon distribution

Fig. 6A–E shows the status of cell regeneration, including observations of its cross-sectional area, intimal growth, axonal cells, and cell density. Based on the results, with the addition of RA, the nerve cross-sectional area increased after 4 weeks, thus implying a rapid recovery of the nerve appearance. When the amount of RA added was 20 mg/kg, the total area of nerve regeneration significantly increased (Fig. 6B). However, no statistically significant differences were found in endoneurium area, axon number, and density (Fig. 6C–E, respectively) in the successfully regenerated nerve cables among the groups.

3.6. Thermal hyperalgesia test

Fig. 7A–C shows the response status of regenerated nerves in radiant photothermal and cold plate tests. According to the results, the addition of RA did not significantly affect the reaction time of the rats and the taxol group and the RA-containing groups, showed similar reaction times. Meanwhile, the results showed similar results to the reaction state of the aforementioned radiation photothermal and cold plate tests. Hence, no significant difference was found in the number of times the rats raised their feet and their reaction time. This result shows that RA

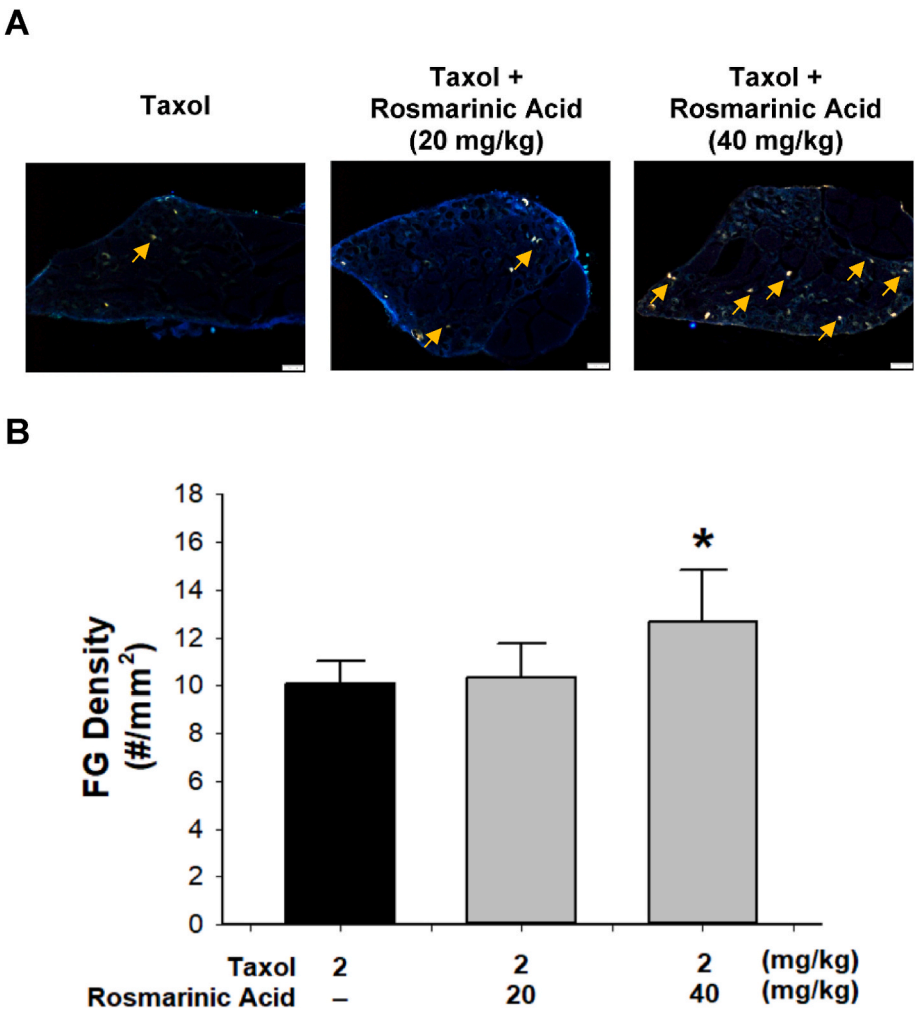

Fig. 5. Effects of RA on FG expression in taxol-treated rats. (A) Representative images of the retrograde axonal tracing with FG (arrows) and (B) comparison for the density of FG expression area in taxol-treated rats. Values are mean ± SEM (n = 10). *$P < 0.05$ compared with taxol group. Scale bar = 100 μm.

did not improve the response status in the early stage of nerve repair.

3.7. Motor coordination test

Fig. 8 shows the result of the motor activity of the regenerated nerve, and the acting ability of the rat is tested by the roller, meanwhile, Fig. 8A and **B** are the activity time and movement speed, respectively. The results showed that the addition of RA did not significantly increase the motor activity of the rats as compared with the taxol group. In addition, the speed and time of the movement had the same trend.

3.8. Electrophysiological detection

Fig. 9 shows the electrophysiological detection and analysis of regenerated nerves. The experiment uses the action potentials obtained from the muscles innervated by the motor nerves to measure the conduction velocity of the motor nerves. The nerve conduction velocity is shown in Fig. 9A. The results show that the taxol group and the groups added with 40 mg/kg RA have statistically significant differences, thereby indicating that RA improves the conduction velocity. Meanwhile, Fig. 9B shows the latency period, and the length of penetration time shows the treatment status of regenerated nerve fibers. The results show that the taxol group has a longer latency period, while the latent period becomes shorter after adding RA. When the RA content is 40 mg/kg, RA has a statistically significant improvement in latency time. In addition, Fig. 9C shows the contraction amplitude of the rat during the detection process. Hence, similar results were shown in the three groups in the contraction test, which showed that the regenerated nerves also recovered slowly in the absence of RA. The MAP area calculated the sum of the amplitude and wave period to evaluate the number of nerve fibers and the recovery. The results are shown in Fig. 9D. The same as the previous test, the three groups had similar results.

4. Discussion

Taxol is an antineoplastic agent that promotes microtubule assembly, which could lead to peripheral neuropathy. A variety of neuroprotective drugs have been developed to reduce the occurrence of neurotoxicity due to taxol.[24] Our previous study demonstrated that taxol could cause worsening of local inflammation and hinder peripheral nerve regeneration.[17] In the present study, we assessed the influence of RA at different doses on regenerating sciatic nerves across a large defect repaired using a silicone rubber conduit in a taxol-treated rat model.

RA was used in this study to facilitate the treatment of nerve defects, and the drug-loaded neural scaffolds were first assessed for biocompatibility *in vitro*. The results from cell culture suggested that RA significantly suppressed NF-κB activity induced by taxol in cells, with good biological properties. In the early stage of nerve injury, distal axons gradually undergo necrosis, forming Wallerian degeneration, and the regulation of histone deacetylase in Schwann cells after injury helps them support the dedifferentiation and redifferentiation processes necessary for each stage of nerve repair, and for accumulation of

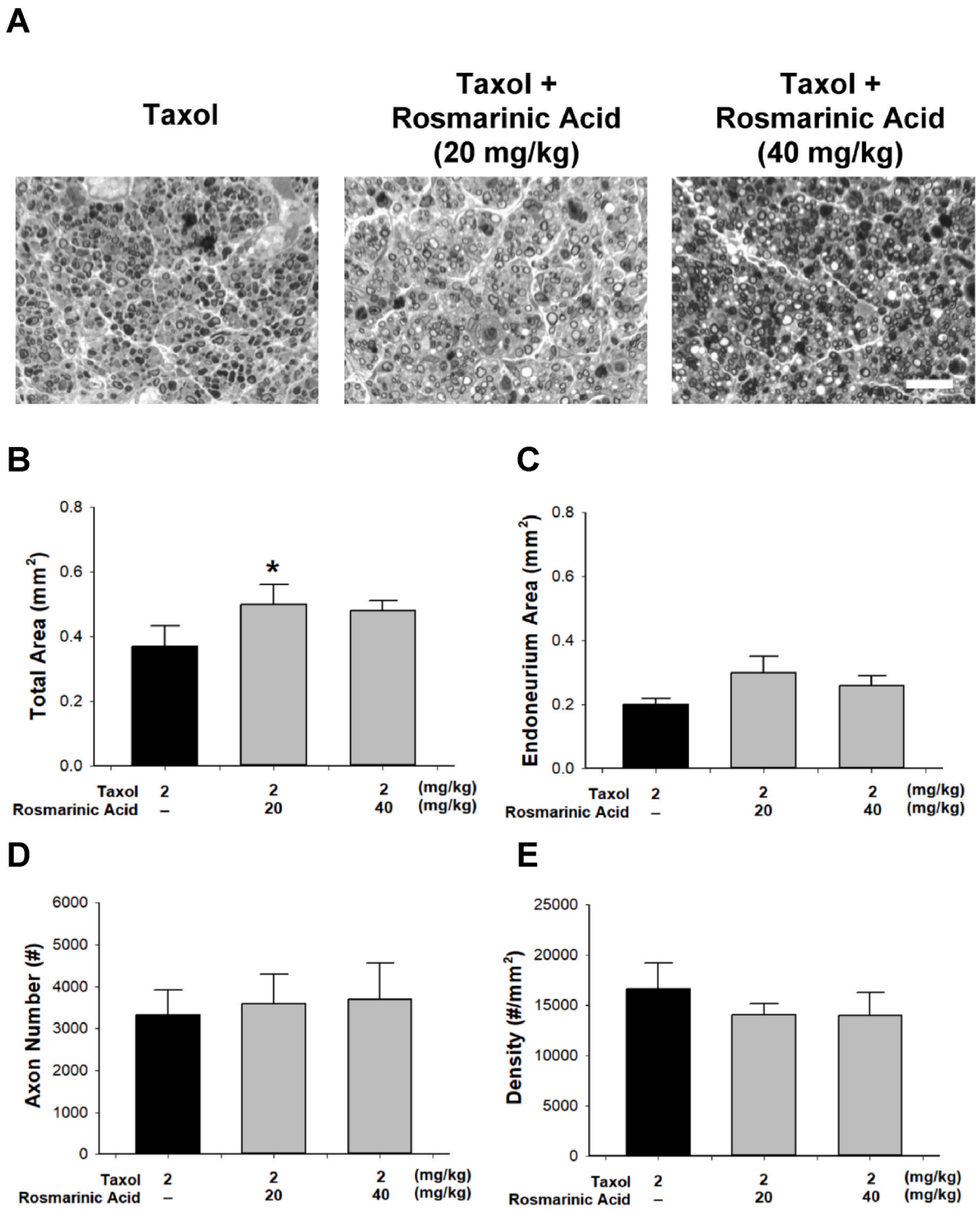

Fig. 6. Effects of RA on axon regeneration in taxol-treated rats. (A) Representative micrographs of nerve tissues and morphometric comparisons including (B) total area, (C) endoneurium area, (D) axon number, and (E) density of regenerated nerves. Values are mean ± SEM (n = 10) *$P < 0.05$ compared with taxol group. Scale bar = 20 μm.

macrophages produces a regulatory effect.[25]

Inflammation of the immune system is a kind of protective response to local injury or infection. The role of inflammation is to eliminate or reduce the source of potentially destructive infections. However, when the inflammation turns persistent and uncontrollable, it can cause damage and harm normal tissues. Excessive inflammation may give rise to excessive local oxidative pressure. The increase in oxidative pressure is due to the imbalance of oxidative metabolism, which leads to the accumulation of non-physiological ROS in cells. Excess ROS forces incensement of lipid peroxidation, resulting in DNA breakage, receiving cell death in succession. This process is the same as the process that taxol causes the oxidative death of neurons, which alters the function of the neuronal cell produces neuropathic pain, or causes the death of residual nerve cells.[10,26]

Immune response is important for axon regeneration at the site of nerve injury, and the activity of macrophages at the site of damaged nerves has an impact on repair. Macrophages, as a variety of cells, can stimulate and inhibit inflammation, promote wound repair, and can

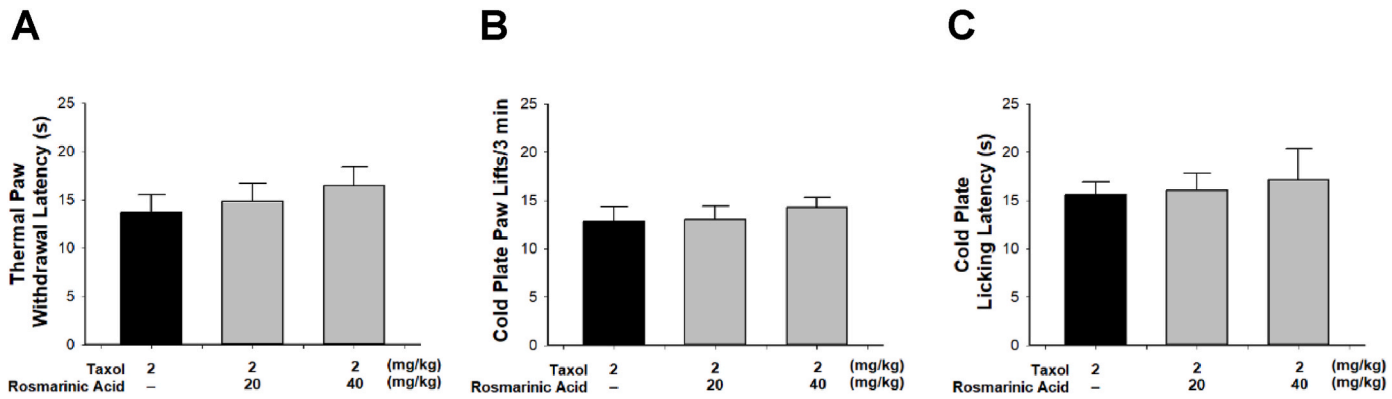

Fig. 7. Analysis of RA with thermal tests, including (A) thermal paw withdrawal, (B) cold plate paw lifts, and (C) cold plate licking latency. Values are mean ± SEM (n = 10) No significant difference was seen among the 3 groups with different doses of RA.

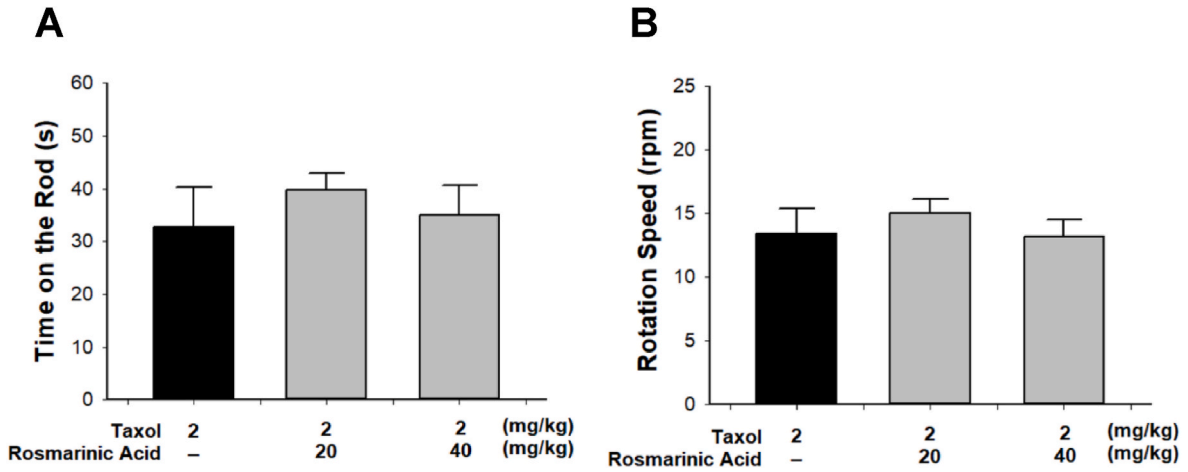

Fig. 8. Analysis of motor coordination tests, including (A) time on the rod and (B) rotation speed tests. Values are mean ± SEM (n = 10) No significant difference was seen among the 3 groups with different doses of RA.

phagocytosis of necrotic cell debris, secretion of growth factors, promotion of angiogenesis and interaction with immune cells. Macrophages are well studied at sites of nerve injury and are critical for nerve regeneration.[27,28] The anti-inflammatory properties of RA have already been confirmed by various studies, and its neuroprotection can promote anti-inflammatory effects, reduce astrogliosis and improve neurotrophic factor expression.[29–31] In addition, RA has the property of inducing the differentiation of activated macrophages, which results in a decrease in the density of macrophages with a lower normative variance when more RA is added.[32]

The results of macrophage infiltration show the changes in macrophages after the addition of RA, and the high density seems to have a synergistic effect with the nerves to achieve a more significant effect. Moreover, CGRP has anti-inflammatory properties and immunomodulatory functions during inflammatory responses, suggesting that the addition of RA is a novel finding in neuroregenerative therapeutic regimens.[33]

Clinically, when treating broken or damaged peripheral nerves, nerve conduits are used to induce nerve cell regeneration and junctions and the addition of drugs can assist nerve growth and achieve the purpose of treatment. Moreover, RA is a water-soluble polyphenolic phytochemical. Substances derived from *Lamiaceae* herbs, comfrey and ferns, and other plants, have antioxidant, anti-inflammatory, anti-apoptotic, anti-tumor, and neuroprotective activities.[34] Meanwhile, the neuroprotective effects of RA may be obtained through its antioxidant and anti-inflammatory effects.[35] RA protected rats from convulsions caused by blood salts by reducing malondialdehyde, a marker of lipid peroxidation, and enhancing the antioxidant defense system.[36] Furthermore, RA inhibited lipopolysaccharide (LPS)-induced upregulation of tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS).[15] In addition, it prevents axonal and myelin disturbances, edema, and inflammatory infiltration in nerves, thereby reducing neuropathic hypersensitivity and protecting nerve tissue.[37]

Based on the applicability of RA, the restoration of neural appearance was first assessed. Although the results of the above studies were not statistically significantly different, the total area of nerve regeneration increased, the intima was thickened, and the density of axonal cell distribution was more uniform with drug addition in the drug-added group. Thus, when nerve regeneration reaches a certain level, the cross-sectional area of the regenerated nerve will first increase and then decrease. Schwann cells undergo mitosis soon after injury, thereby forming continuous Schwann cell columns wrapped by basement membranes, which are located in axons. It acts as a guide and nutritional support during regeneration. After some time, the Schwann cell column will reduce to 10%–20% of its original diameter because of collagen contraction.[38] Therefore, it can be estimated that after the initial injury of the nerve in this study, the rapid division of Schwann leads to an increase in the cross-sectional area of the regenerated nerve, and with the addition of RA, the phenomenon of inflammation and edema is improved, and then the distribution of axonal cells is affected.

When a nerve is injured, the factors affecting the recovery of nerve function mainly depend on the degree of recovery of the axon itself. The integrity of the basal layer and the time it takes to regenerate to its original location will affect the recovery of nerve function, that is, after

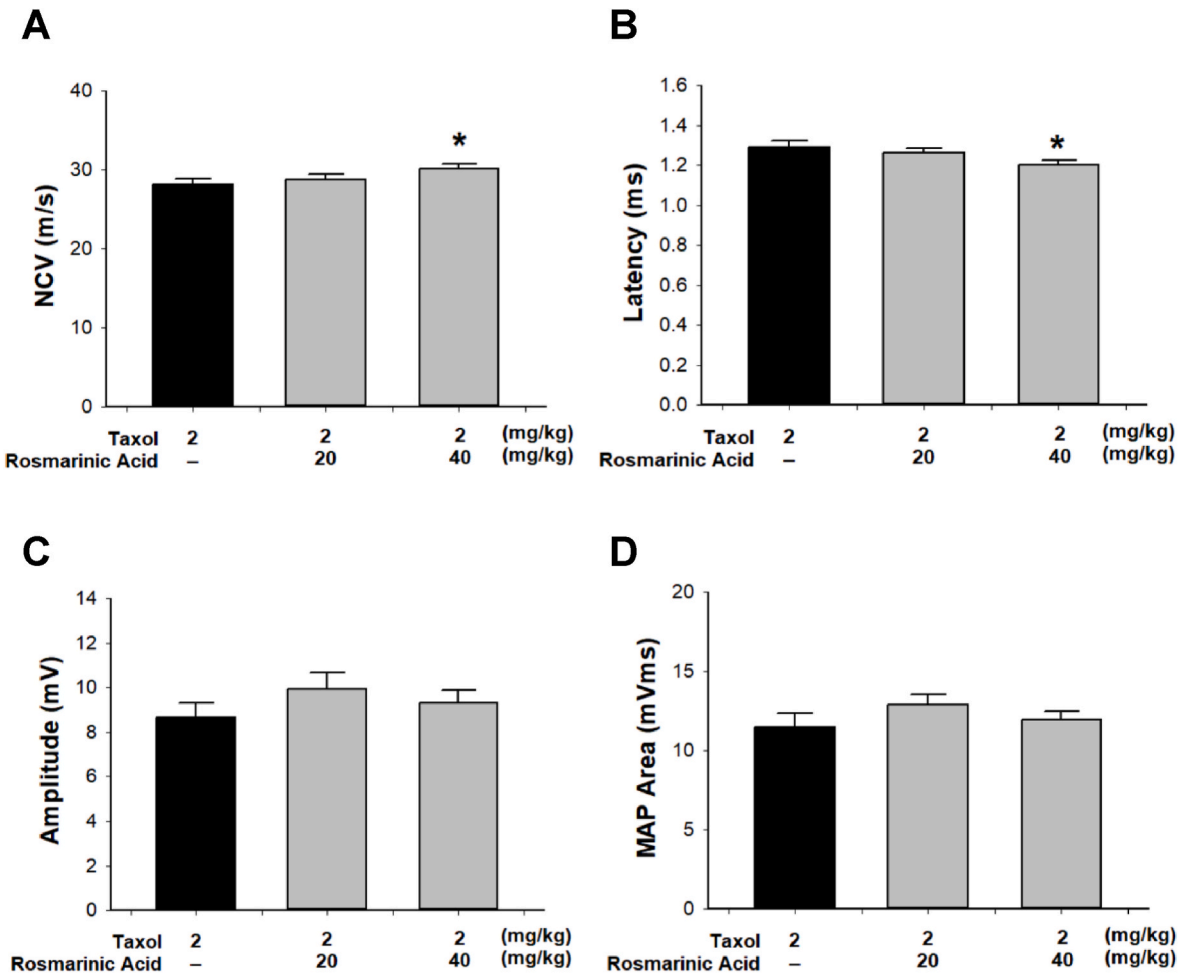

Fig. 9. Electrophysiological analysis of regenerated nerves in taxol-treated rats. Analysis of evoked MAPs, including (A) NCV, (B) latency, (C) peak amplitude, and (D) area under the MAP curves. Values are mean ± SEM (n = 10) *$P < 0.05$ compared with taxol group.

nerve rupture. In addition, when the muscle has not been regenerated to its original distribution, the muscle will completely atrophy because it cannot receive nerve stimulation. In general, the functional recovery time of regenerated nerves is about 6–12 months. Therefore, although the initial repair has been achieved in appearance, its effect on muscles still needs to be investigated.

The results of the motor coordination test also show that the standard differences in the taxol group are more dispersed, which shows that the activity of each group of rats is not uniform, and the group added with the drug has a more stable motor activity performance. Neuropathic pain is a kind of pain caused by trauma or disease of the peripheral or central nervous system. In the physiological mechanism of nerves, nociceptive pain is caused by tissue damage. Therefore, the addition of RA may induce a potential mechanism for reducing pain, which in turn makes the rat exercise. Increased activity. Based on the reference, inflammation of nerves is related to the induction and maintenance of neuropathic pain, and RA has antioxidant and anti-inflammatory properties, which play a key role in the treatment process because RA has peripheral and central analgesia in addition to anti-inflammatory properties.[39] Furthermore, the addition of RA reduces the stimulation of adjacent motor neurons to form toxic substances, which in turn reduces motor neuron loss, improves motor performance, inhibits weight loss, and prolongs survival in rats.[35]

The electrophysiological tests showed that during the healing process of the regenerated nerve after injury, the appearance, muscle movement, and reaction ability of the control group all had initial functional recovery. However, adding RA can improve the conduction velocity of the regenerated nerve. This is because RA is a powerful antioxidant with direct free radical scavenging properties, able to protect neurons by coordinating precise signaling pathways and neurotransmitter manipulation.[40] Based on the data, the addition of RA led to an increase in the cross-sectional area and intimal thickness of the regenerated nerve fibers, as well as the uniform distribution of axonal cells made the rats have better muscle response after stimulation. Herein, the electrical conduction velocity was confirmed.

We used a silicone rubber tube as a physical guide for regenerating axons, providing a protected environment for nerve regeneration. Silicone is a material with good biocompatibility and is widely used in various medical devices. Since the diffusion rate of substances in silicone is very stable, it is used for drug-carrying treatment. It will be a new research direction to combine silicone with drugs for nerve regeneration. However, we also acknowledged the limitations of silicone conduit, such as the lack of internal structure, the risk of fibrosis and neuroma formation, and the need for surgical removal after nerve regeneration.[41,42] Therefore, we suggested that future studies should explore the use of biodegradable materials or scaffolds with internal guidance channels to enhance the efficacy of drugs for peripheral nerve regeneration.

Our study has certain limitations. First, in this study, we focused on evaluating CD68 (as a marker for various cells of the macrophage lineage) and the anti-inflammatory activity of RA in a cell culture model. We did not further measure various inflammatory and oxidative biomarkers in the biological samples of rats. Thus, we suggest that future studies measure various inflammatory and oxidative biomarkers in the

biological samples of rats to further validate the anti-inflammatory and antioxidative activities of RA *in vivo*. Additionally, we did not compare the effects of RA administered through other routes, such as oral or intravenous. Our approach exclusively involved employing an IP drug delivery system for RA, utilizing the silicone rubber conduit as a physical guide. Nevertheless, the integration of silicone with RA for nerve regeneration represents a novel research direction, and we recommend further studies to optimize the dosage and delivery method of RA.

5. Conclusions

This study investigated the regenerated nerve after RA treatment. The antioxidant activity of RA is related to the potential mechanism of the neuroprotective effect. The results after adding RA showed that the regenerated nerve fiber could suppress the inflammatory response during the short-term recovery period so that the regenerated nerve grows well. The use of RA as a drug to regenerate nerve growth is worthy of research and follow-up long-term experiments. However, before RA can be co-administered with taxol in the clinic, further studies are needed, including dose-escalation studies, long-term toxicity studies, and clinical trials.

Author contributions

Conceptualization: Yueh-Sheng Chen and Chung-Chia Chen. Experimental Investigation: Ping-Ling Chiu, Mei-Chen Lin, and Shih-Tien Hsu. Data Analysis and Interpretation: All Authors. Manuscript Preparation: All Authors. Ping-Ling Chiu, Mei-Chen Lin, and Shih-Tien Hsu contributed equally to the work.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare that they have no conflict of interest. In the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Acknowledgments

This research project was financially supported by Taichung Veterans General Hospital (TCVGH-1106402B), Taiwan; Taipei City Hospital (TPCH-108-63), Taiwan; National Science and Technology Council (NSTC 111-2314-B-039-086), Taiwan; and China Medical University (CMU110-MF-94), Taiwan.

References

1. Sabaratnam V, Kah-Hui W, Naidu M, Rosie David P. Neuronal health - can culinary and medicinal mushrooms help? *J Tradit Complement Med*. 2013;3(1):62–68.
2. Sivaramakrishnan G, Sridharan K. Electrical nerve stimulation for xerostomia: a meta-analysis of randomised controlled trials. *J Tradit Complement Med*. 2017;7(4):409–413.
3. Navarro X, Vivo M, Valero-Cabre A. Neural plasticity after peripheral nerve injury and regeneration. *Prog Neurobiol*. 2007;82(4):163–201.
4. Girard C, Liu S, Cadepond F, et al. Etifoxine improves peripheral nerve regeneration and functional recovery. *Proc Natl Acad Sci USA*. 2008;105(51):20505–20510.
5. Mi D, Yuan Y, Zhang Y, et al. Injection of Fluoro-Gold into the tibial nerve leads to prolonged but reversible functional deficits in rats. *Sci Rep*. 2019;9(1):9906.
6. Xu B, Zhang WS, Yang JL, Xu H, Deng XM, Zhang YQ. Dexmedetomidine blocks thermal hyperalgesia and spinal glial activation in rat model of monoarthritis. *Acta Pharmacol Sin*. 2010;31(5):523–530.
7. Ahn SH, Chang IA, Kim KJ, Kim CJ, Namgung U, Cho CS. Bogijetong decoction and its active herbal components protect the peripheral nerve from damage caused by taxol or nerve crush. *BMC Compl Alternative Med*. 2016;16.
8. Cavaletti G, Tredici G, Braga M, Tazzari S. Experimental peripheral neuropathy induced in adult-rats by repeated intraperitoneal administration of taxol. *Exp Neurol*. 1995;133(1):64–72.
9. Dwivedi P, Han SY, Mangrio F, et al. Engineered multifunctional biodegradable hybrid microparticles for paclitaxel delivery in cancer therapy. *Mat Sci Eng C-Mater*. 2019;102:113–123.
10. Jang HJ, Hwang S, Cho KY, Kim DK, Chay KO, Kim JK. Taxol induces oxidative neuronal cell death by enhancing the activity of NADPH oxidase in mouse cortical cultures. *Neurosci Lett*. 2008;443(1):17–22.
11. Schiff PB, Fant J, Horwitz SB. Promotion of microtubule assembly invitro by taxol. *Nature*. 1979;277(5698):665–667.
12. Areti A, Komirishetty P, Kalvala AK, Nellaiappan K, Kumar A. Rosmarinic acid mitigates mitochondrial dysfunction and spinal glial activation in oxaliplatin-induced peripheral neuropathy. *Mol Neurobiol*. 2018;55(9):7463–7475.
13. Fachel FNS, Schuh RS, Veras KS, et al. An overview of the neuroprotective potential of rosmarinic acid and its association with nanotechnology-based delivery systems: a novel approach to treating neurodegenerative disorders. *Neurochem Int*. 2019;122:47–58.
14. Ma ZJ, Lu YB, Yang FG, et al. Rosmarinic acid exerts a neuroprotective effect on spinal cord injury by suppressing oxidative stress and inflammation via modulating the Nrf2/HO-1 and TLR4/NF-kappa B pathways. *Toxicol Appl Pharmacol*. 2020:397.
15. Rahbardar MG, Amin B, Mehri S, Mirnajafi-Zadeh SJ, Hosseinzadeh H. Rosmarinic acid attenuates development and existing pain in a rat model of neuropathic pain: an evidence of anti-oxidative and anti-inflammatory effects. *Phytomedicine*. 2018;40:59–67.
16. Wu BY, Liu CT, Su YL, Chen SY, Chen YH, Tsai MY. A review of complementary therapies with medicinal plants for chemotherapy-induced peripheral neuropathy. *Compl Ther Med*. 2019;42:226–232.
17. Hsu ST, Yao CH, Hsu YM, Lin JH, Chen YH, Chen YS. Effects of taxol on regeneration in a rat sciatic nerve transection model. *Sci Rep*. 2017;7.
18. Liao CF, Hsu ST, Chen CC, et al. Effects of electrical stimulation on peripheral nerve regeneration in a silicone rubber conduit in taxol-treated rats. *Materials*. 2020;13(5).
19. Baudy AR, Saxena N, Gordish H, Hoffman EP, Nagaraju K. A robust in vitro screening assay to identify NF-kappaB inhibitors for inflammatory muscle diseases. *Int Immunopharm*. 2009;9(10):1209–1214.
20. Hsu ST, Yao CH, Hsu YM, Lin JH, Chen YH, Chen YS. Effects of taxol on regeneration in a rat sciatic nerve transection model. *Sci Rep*. 2017;7, 42280.
21. Liao CF, Hsu ST, Chen CC, et al. Effects of electrical stimulation on peripheral nerve regeneration in a silicone rubber conduit in taxol-treated rats. *Materials*. 2020;13(5).
22. Liu WL, Chang JM, Chong IW, et al. Curcumin inhibits LIN-28a through the activation of miRNA-98 in the lung cancer cell line A549. *Molecules*. 2017;22(6).
23. Su HF, Samsamshariat A, Fu J, et al. Oleylethanolamide activates Ras-Erk pathway and improves myocardial function in doxorubicin-induced heart failure. *Endocrinology*. 2006;147(2):827–834.
24. Scripture CD, Figg WD, Sparreboom A. Peripheral neuropathy induced by paclitaxel: recent insights and future perspectives. *Curr Neuropharmacol*. 2006;4(2):165–172.
25. Yang DP, Zhang DP, Mak KS, Bonder DE, Pomeroy SL, Kim HA. Schwann cell proliferation during Wallerian degeneration is not necessary for regeneration and remyelination of the peripheral nerves: axon-dependent removal of newly generated Schwann cells by apoptosis. *Mol Cell Neurosci*. 2008;38(1):80–88.
26. Deuis JR, Dvorakova LS, Vetter I. Methods used to evaluate pain behaviors in rodents. *Front Mol Neurosci*. 2017;10.
27. Khamse S, Sadr SS, Roghani M, Hasanzadeh G, Mohammadian M. Rosmarinic acid exerts a neuroprotective effect in the kainate rat model of temporal lobe epilepsy: underlying mechanisms. *Pharm Biol*. 2015;53(12):1818–1825.
28. Lundborg G, Dahlin LB, Danielsen N, Nachemson AK. Tissue-specificity in nerve regeneration. *Scand J Plast ReConstr Surg Hand Surg*. 1986;20(3):279–283.
29. Fachel FNS, Michels LR, Azambuja JH, et al. Chitosan-coated rosmarinic acid nanoemulsion nasal administration protects against LPS-induced memory deficit, neuroinflammation, and oxidative stress in Wistar rats. *Neurochem Int*. 2020:141.
30. Moon DO, Kim MO, Lee JD, Choi YH, Kim GY. Rosmarinic acid sensitizes cell death through suppression of TNF-alpha-induced NF-kappa B activation and ROS generation in human leukemia U937 cells. *Cancer Lett*. 2010;288(2):183–191.
31. Fonteles AA, de Souza CM, Neves JCD, et al. Rosmarinic acid prevents against memory deficits in ischemic mice. *Behav Brain Res*. 2016;297:91–103.
32. Heo SK, Noh EK, Yoon DJ, et al. Rosmarinic acid potentiates ATRA-induced macrophage differentiation in acute promyelocytic leukemia NB4 cells. *Eur J Pharmacol*. 2015;747:36–44.
33. Holzmann B. Antiinflammatory activities of CGRP modulating innate immune responses in health and disease. *Curr Protein Pept Sci*. 2013;14(4):268–274.
34. Airoldi C, Sironi E, Dias C, et al. Natural compounds against Alzheimer's disease: molecular recognition of A beta 1-42 peptide by salvia sclareoides extract and its major component, rosmarinic acid, as investigated by NMR. *Chem Asian J*. 2013;8(3):596–602.
35. Shimojo Y, Kosaka K, Noda Y, Shimizu T, Shirasawa T. Effect of rosmarinic acid in motor dysfunction and life span in a mouse model of familial amyotrophic lateral sclerosis. *J Neurosci Res*. 2010;88(4):896–904.
36. Rahbardar MG, Hosseinzadeh H. Effects of rosmarinic acid on nervous system disorders: an updated review. *Naunyn-Schmiedeberg's Arch Pharmacol*. 2020;393(10):1779–1795.
37. Shimojo Y, Kosaka K, Noda Y, Shimizu T, Shirasawa T. Effect of rosmarinic acid in motor dysfunction and life span in a mouse model of familial amyotrophic lateral sclerosis. *J Neurosci Res*. 2010;88(4):896–904.
38. Pellegrino RG, Spencer PS. Schwann-cell mitosis in response to regenerating peripheral axons invivo. *Brain Res*. 1985;341(1):16–25.

39. Nadeem M, Imran M, Gondal TA, et al. Therapeutic potential of rosmarinic acid: a comprehensive review. *Appl Sci-Basel.* 2019;9(15).
40. Lee HJ, Cho HS, Park E, et al. Rosmarinic acid protects human dopaminergic neuronal cells against hydrogen peroxide-induced apoptosis. *Toxicology*. 2008;250 (2-3):109–115.
41. Cavaletti G, Tredici G, Braga M, Tazzari S. Experimental peripheral neuropathy induced in adult rats by repeated intraperitoneal administration of taxol. *Exp Neurol.* 1995;133(1):64–72.
42. Dwivedi P, Han S, Mangrio F, et al. Engineered multifunctional biodegradable hybrid microparticles for paclitaxel delivery in cancer therapy. *Mater Sci Eng C Mater Biol Appl.* 2019;102:113–123.