Therapeutic Ultrasound and Treadmill Training Suppress Peripheral Nerve Injury–Induced Pain in Rats

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Background. Although evidence suggests that therapeutic ultrasound (TU) in combination with treadmill training (TT) suppresses nerve injury–associated pain, the molecular mechanisms for this action are not clear.

Objective. The purpose of this research was to study the possible beneficial effects of TU and TT, alone and in combination, on 2 clinical indicators of neuropathic pain and correlate these findings with changes in inflammatory mediators within the spinal cord. Our experimental model used the well-known chronic constriction injury (CCI) of the rat sciatic nerve.

Design. This was an experimental study.

Methods. Each group contained 10 rats. Group 1 underwent only the CCI procedure. Group 2 underwent a sham operation where the sciatic nerve was exposed but not ligated. Group 3 had the sham operation followed by both TT and TU. Groups 4, 5, and 6 underwent the CCI procedure followed by TT alone, TU alone, and both the TT and TU interventions, respectively. Heat and mechanical sensitivity, interleukin-6 (IL-6), interleukin-10 (IL-10), and ionized calcium binding adaptor molecule 1 (Iba1) were evaluated.

Results. Compared with group 1 animals, TT or TU, or both, produced smaller decreases in mechanical withdrawal threshold and heat withdrawal latencies. The combination of TT and TU was more effective than either treatment alone. In addition, rats that received these treatments did not express the upregulation of IL-6 and Iba1 in their spinal cords on postoperative days 14 and 28, as was found in the group 1 animals.

Limitations. These experimental findings may not be generalizable to humans.

Conclusions. The combination of TU and TT reduces neuropathic pain more than either modality alone. This beneficial effect appears related to downregulation of proinflammatory IL-6 and Iba1, while upregulating the anti-inflammatory IL-10.

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Peripheral nerve injury commonly leads to neuropathic pain characterized by thermal hyperalgesia, mechanical allodynia, spontaneous pain, and mechano-hyperalgesia.[1] Peripheral and central sensitization of nociceptive pathways are crucial to the onset and development of neuropathic pain.[1,2] Glial dysfunction and the increased levels of proinflammatory cytokines are 2 predominant mechanisms by which glial cells intensify neuronal activities in the spinal dorsal horn.[3,4] However, effective management of neuropathic pain remains a clinical challenge. Therapeutic ultrasound (TU) has been reported to be useful in the treatment of carpal tunnel syndrome, the most common compression neuropathy in humans.[5,6] Moreover, treadmill training (TT) alleviated peripheral neuropathic pain in preclinical rodent studies.[7] Currently, there are few treatments that have been shown to be effective in treating symptoms arising from nerve injury, and several pharmacotherapies used for treating neuropathic pain unfortunately are associated with adverse side effects.[8–10] However, to our knowledge, no studies have examined whether combining TU and exercise can be more effective than performing TU or exercise alone.

Recently, it has been shown that TU at an intensity of 0.5 W/cm^2 recovers up to 90% of the sciatic functional index from the sciatic nerve crush injury in mice.[11] Furthermore, TU with the intensities of 0.3 and 0.4 W/cm^2 induces a long-acting anti-nociceptive effect on an experimental rat model of trigeminal neuropathic pain.[12] Additionally, there is a growing body of evidence that forced exercise suppresses many types of chronic pain, including peripheral neuropathic pain,[7,13] diabetic neuropathic pain,[14,15] postoperative pain,[16,17] and chronic muscle pain[18] in rodents.

In addition, the native immune cells (ie, microglial cells) in the central nervous system play critical roles in the immune responses following nerve damage and are greatly associated with chronic pain.[19–21] Evidence has demonstrated that the activated microglia and the microglial marker (ionized calcium-binding adaptor molecule 1 [Iba1]) markedly increased in the spinal cord following peripheral nerve injury.[22] Additionally, it has been shown that the cytokines (ie, interleukin-6 [IL-6]) were implicated in the macrophages and microglial cells in the spinal cord after the nerve lesion.[23–25] Furthermore, there is increasing appreciation for the cytokines' ability to directly affect the excitatory neuronal transmission, which results in pain facilitation and spontaneous activity.[26,27]

Following this further, our previous study[7] showed that TT attenuated mechanical allodynia and thermal hyperalgesia following chronic constriction injury (CCI) in rats. Furthermore, these behavioral (clinical) improvements were associated with suppressed expression of proinflammatory cytokines in the sciatic nerve. The tissue inflammation or nerve damage is implicated in various mediator releases in the spinal cord, resulting in pain hypersensitivity. Thus, the purpose of this experiment was to estimate the impacts of TU, TT, or a combination (TU+TT) on responses directed toward (1) the heat and mechanical stimuli and (2) expression of Iba1, IL-6, and interleukin-10 (IL-10) in the spinal cord by using a constricted-based (CCI) animal model of neuropathic pain.

Materials and Methods

Animals

Effort was made to minimize the discomfort and decrease the number of experimental animals. All experiments were conducted according to the International Association for the Study of Pain ethical guidelines.[28] The experiments were performed on male Sprague-Dawley rats (National Laboratory Animal Center, Taipei, Taiwan) weighing 220 to 270 g. Animals were kept in a climate-controlled room maintained at 22°C with approximately 50% relative humidity in the animal housing facility of National Cheng Kung University. They inhabited a 12-hour light-dark cycle (light on at 6:00 am), and food and water were available ad libitum to them until the time of the experiments.

Groups and Design

Sixty rats were randomly and blindly separated into 6 groups (n=10 per group). Group 1 underwent CCI surgery but received no other intervention. Group 2 underwent a sham operation where the sciatic nerve was exposed but not ligated. Group 3 also had the sham operation followed by both TT and TU as described later. Group 4 underwent the CCI procedure followed by TT alone. Group 5 underwent the CCI procedure followed by TU alone. Group 6 underwent the CCI procedure followed by both the TT and TU interventions.

Beginning on postoperative day 3 and continuing 5 days a week for the next 4 weeks, groups 3 and 6 had TU for 5 minutes followed by 30 minutes of TT. During this same time frame, group 4 had TT alone for 30 minutes, and group 5 had TU alone for 5 minutes. In our previous study,[7] neuropathic pain developed in rats 3 days after the animals had undergone the CCI damage and lasted for up to 30 days.

For the neurobehavioral assessments, the rats were evaluated twice for the mechanical withdrawal threshold and heat withdrawal latency on the day prior to surgery, and these 2 measurements were averaged to gain a sole baseline of mechanical withdrawal threshold and heat withdrawal latency for each evaluation. Subsequently, these rats were evaluated on postoperative days 3, 7, 14, 21, and 28. Their spinal cords (L4–L5) were extracted for IL-6, IL-10, and Iba1 analyses on postoperative day 28. Some rats without behavioral evaluations were killed to obtain spinal cords (L4–L5) for IL-6, IL-10, and Iba1 analyses on postoperative day 14.

CCI Procedure

After animals were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally), 4 ligatures, surrounded by 4 chromic gut sutures, were tied around the sciatic nerve as described by Bennett and Xie.[29] Using 4× magnification to assist in this procedure, the ties were tightened so that the diameter of the nerve was seen to be barely constricted. This tightening of the ties sometimes produced a small brief twitch of the surrounding muscles. After the CCI surgery, the skin incision was closed with

wound clips, and the rat was returned to its cage for recovery.

Heat and Mechanical Sensitivity

One experienced person, blinded to which group an animal belonged, performed all of the neurobehavioral examinations. For consistency, the neurobehavioral assessments were performed between 9:00 and 11:00 am. The animals were evaluated for the mechanical withdrawal threshold and heat withdrawal latency after a period of 5 to 7 days of adapting to the test environment and experimenters.

The heat withdrawal latency was examined according to the method of Hargreaves et al.[30] In brief, the lateral plantar area of the hind paw was exposed to a constant-intensity radiant heat source by the Hargreaves plantar test device (Ugo Basile, Comerio, Italy). After the rat withdrew its hind paw, the paw withdrawal latency (in seconds) was recorded. The cutoff time was set at 20 seconds to avoid tissue injury.[15] To measure the mechanical withdrawal threshold, the animals were put separately in a clear, plexiglass conditioning chamber above a wire-mesh floor. Each von Frey hair filament (anesthesiometer, Somedic AB, Hörby, Sweden) was touched to the lateral plantar surface of the rat hind paw for 3 seconds. This paw withdrawal threshold (in grams) was verified by testing with the next thicker von Frey hair filament, which always evoked paw withdrawal.[7,15]

Application of TU

After the animals were lightly anesthetized with isoflurane (1%), a TU device (US-750, Bunkyo-ku, Tokyo, Japan) was used while the transducer was applied to the skin of rats with gel. The parameters were 1 MHz with pulse (20% duty cycle), 1-W/cm^2 intensity, and 100-Hz frequency (beam nonuniformity ratio=3.6) for 5 minutes a day. To prevent damage by cavitation, the S-size ultrasound probe was used in pulsed mode and applied with a gentle circular movement applied over the skin incision.

TT Procedure

Treadmill speed was set at 14 to 16 m/min with an 8% incline for 30 minutes (Treadmill Exerciser T510, Diagnostic and Research Instruments, Singa, Taiwan).[31] Rats were exercised 5 days a week for 4 weeks. When necessary, hind limbs were gently prodded to maintain running adherence.

Cytokine Analysis

Animals were sacrificed, using pentobarbital sodium (200 mg/kg, intraperitoneally), on postoperative day 14 or 28. After adding homogenization buffer (4°C), tissue samples were homogenized and centrifuged for preparing the protein assay. The protein concentrations in the supernatant were quantified by the Lowry protein assay, in which each plate was inserted into a plate reader (Molecular Devices LLC, Specification 383, Sunnyvale, California) to read the optical density of each well at an absorbance of 750 nm.[16,32] These concentrations of IL-6 and IL-10 in the supernatant were detected using the DuoSet ELISA Development Kit (R&D Systems, Minneapolis, Minnesota).[17,33] Our investigative protocols were practiced according to the manufacturer's recommended procedures.

Immunohistochemistry

Initially, the spinal cords (L4–L5) were removed and embedded in a cryo-embedding media and were cut into 25-μm-thick coronal sections. Every 12th section, for a total of 15 sections, was analyzed, and the results were averaged for each animal. The immunohistochemistry procedure was performed as previously reported.[34] In brief, after overnight incubation at room temperature with anti-Iba1 antibody (1:1,000, Wako Pure Chemical Industries Ltd, Osaka, Japan), the spinal cord sections were incubated at room temperature with biotinylated goat anti-rabbit IgG antibodies (1:1,000, Vector Laboratories Inc, Burlingame, California) for 2 hours. Then, the sample sections were placed on glass slides and were observed under a microscope (Axioskop 2 Plus, Carl Zeiss AG, Oberkochen, Germany). Each immune-stained section showed a representative expression of immunoreactivity that was photographed at 2.5× magnification. After each of them was imported into NIH Image J 1.42q software (National Institutes of Health, Bethesda, Maryland), all of the images underwent color deconvolution to distinguish the diaminobenzidine reaction product from hematoxylin counterstain.[34]

Data Analysis

The data are shown as the mean (±standard error of the mean) of the number of observations unless noted otherwise and were analyzed by 1-way or 2-way analysis of variance (ANOVA) for repeated measures. Alpha=.05 is indicated as the significance threshold for ANOVA, and the same value (alpha=.05) was noted as the threshold for assessing post hoc significance (after Bonferroni correction). The Statistical Package for the Social Sciences (SPSS for Windows, version 17.0, SPSS Inc, Chicago, Illinois) was used for the statistical analyses.

Role of the Funding Source

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Results

TU and TT Suppress the Progress of CCI-Associated Mechanical Allodynia and Thermal Hyperalgesia

As shown in Figure 1, baseline values for mechanical withdrawal threshold (Fig. 1A) and thermal withdrawal latency (Fig. 1B) were not significantly different among the 6 groups (2-way repeated-measures ANOVA). On postoperative day 3, however, mechanical thresholds and thermal latencies were significantly lower than at baseline in all groups except for groups 2 and 3, the 2 groups that underwent the sham operation.

By postoperative day 14, groups 4, 5, and 6 (the CCI groups that also received TT, TU, or TT+TU, respectively) showed rising mechanical withdrawal thresholds and thermal withdrawal latencies. These findings were different from those in group 1 (the worst-case scenario group that had only the CCI operation), where mechanical withdrawal threshold (Fig. 1A) and thermal withdrawal latency (Fig. 1B) both remained significantly lower than at baseline and lower than in the other groups.

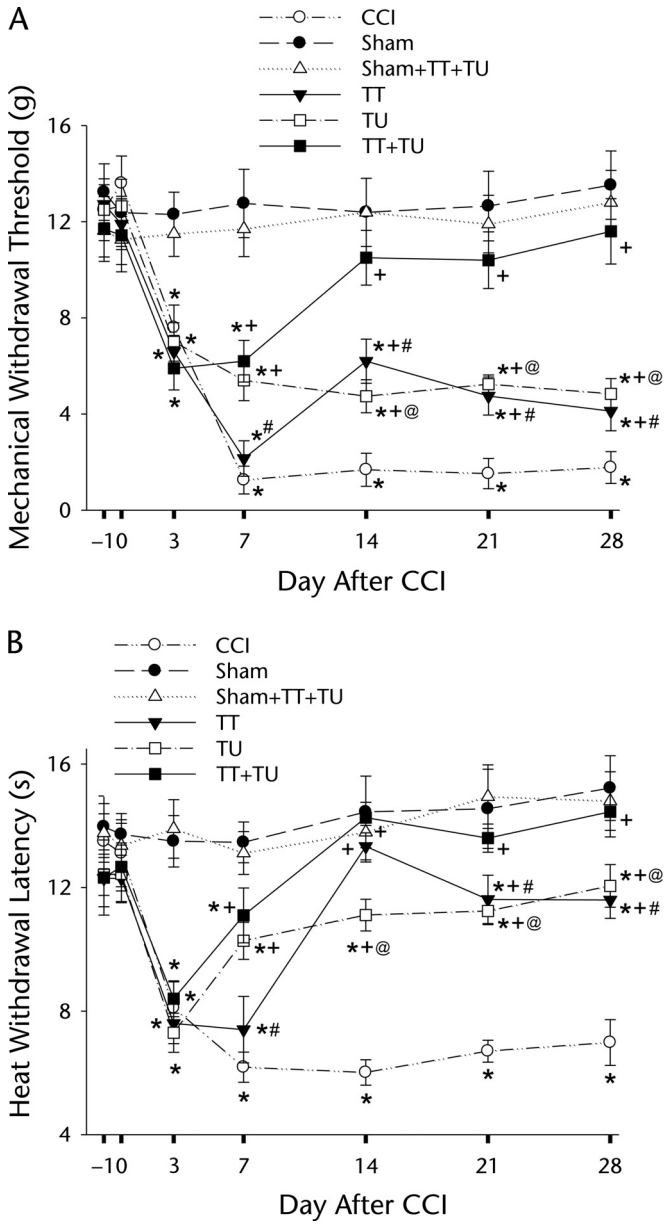

Figure 1.
The behavioral time courses of a (A) mechanical withdrawal threshold and (B) thermal withdrawal latency (B) in the CCI, sham, sham+TT+TU, TT, TU, and TT+TU rats, where CCI=chronic constriction injury, sham=sham operated, sham+TT+TU=sham operated rats that received treadmill training and therapeutic ultrasound, TT=CCI rats that underwent treadmill training, TU=CCI rats that received therapeutic ultrasound, and TT+TU=CCI rats that received treadmill training and therapeutic ultrasound. The data are presented as the mean (±standard error of the mean) for 10 rats per group. $^{*}P<.05$ compared with the sham group, $^{+}P<.05$ compared with the CCI group, $^{\#}P<.05$ when the TT+TU group was compared with the TT group, $^{@}P<.05$ when the TT+TU group was compared with the TU group (2-way analysis of variance for repeated measures followed by post hoc Bonferroni test).

By postoperative day 28, groups 2 and 3 (the sham-operated groups) and group 6 (the CCI group that received TT+TU) were not significantly different from baseline or from each other. In contrast, group 1 continued to show no improvements in either mechanical threshold or withdrawal latency.

The mechanical withdrawal thresholds and heat withdrawal latencies in the 6 groups (Figs. 1A and 1B) were analyzed using a 2-way repeated-measures ANOVA and demonstrated significant main effects for groups ($F_{5,54}=38.55$, $P<.0001$; $F_{5,54}=40.23$, $P<.0001$), time ($F_{6,324}=41.87$, $P<.0001$; $F_{6,324}=51.69$, $P<.0001$), and significant interactions ($F_{30,324}=7.91$, $P<.0001$; $F_{30,324}=7.66$, $P<.0001$), respectively. The post hoc comparisons showed no significant differences between groups 2 (sham operation only) and 3 (sham operation+TT+TU) for mechanical withdrawal threshold ($P>.55$) and heat withdrawal latency ($P>.46$), whereas post hoc comparisons exhibited significant differences between group 4 (or groups 5 and 6) and group 1 (or groups 2 and 3) ($P<.01$, Fig. 1A; $P<.01$, Fig. 1B), respectively.

TU and TT Inhibit Expression of IL-6 but Increase the Expression of IL-10

The expression of IL-6 (a proinflammatory cytokine) in the spinal cord was significantly increased in group 1 (CCI surgery only) compared with group 2 (sham operation) or group 3 (sham operation+TT+TU) on postoperative days 14 and 28 (all $P<.01$, Fig. 2A). By comparison, group 4 (CCI surgery+TT), group 5 (CCI surgery+TU), and group 6 (CCI surgery+TT+TU) showed significantly lower IL-6 expression than group 1 on postoperative days 14 and 28 (all $P<.008$, Fig. 2A).

The expression of IL-10 (an anti-inflammatory cytokine) in the spinal cord was not significantly different among the 6 groups on postoperative day 14 (all $P>.58$, Fig. 2B). However, on postoperative day 28, groups 2, 4, 5, and 6 had significantly more IL-10 compared with group 1 (all $P<.01$, Fig. 2B). There were no significant differences in either IL-6 or IL-10 expression between groups 2 and 3, the 2 sham-operated groups.

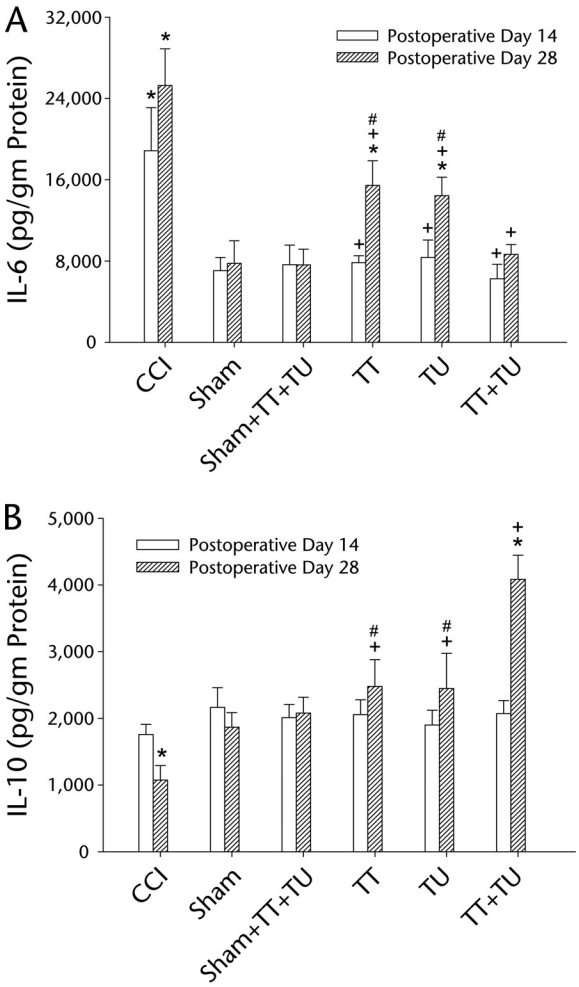

Figure 2.
The levels of (A) interleukin-6 (IL-6) and (B) interleukin-10 (IL-10) on days 14 and 28 after CCI in the spinal cord of 5 different groups of rats: CCI, sham, sham+TT+TU, TT, TU, and TT+TU, where CCI=chronic constriction injury, sham=sham operated, sham+TT+TU=sham operated rats that received treadmill training and therapeutic ultrasound, TT=CCI rats that underwent treadmill training, TU=CCI rats that received therapeutic ultrasound, and TT+TU=CCI rats that received treadmill training and therapeutic ultrasound. The values are presented as the mean (±standard error of the mean) for 7 rats per group. $^{*}P<.05$ compared with the sham group, $^{+}P<.05$ compared with the CCI group, $^{\#}P<.05$ when the TT+TU group was compared with the TU or TT group (1-way analysis of variance followed by post hoc Bonferroni test).

TU and TT Prevent the Upregulation of Iba1 Immunohistochemistry (Iba1 IR) in Rat Spinal Cord After CCI Injury

Photomicrographs of Iba1 reactive cells in the rat spinal cords harvested on postoperative day 28 are shown in Figure 3. The percent of area of Iba1 was significantly increased in group 1 compared with group 2 on postoperative days 14 and 28 (all $P<.01$, Fig. 3G). On postoperative days 14 and 28, the 3 therapeutic groups (groups 4–6) showed a reduction in the percent of area of Iba1 compared with group 1 (all $P<.01$, Fig. 3G). Within the 3 treated groups, group 6 showed the lowest percent of area of spinal Iba1 compared with groups 4 and 5 ($P<.01$, Fig. 3G). Lastly, there was no significant difference in Iba1 areas between the sham-operated groups (groups 2 and 3).

Discussion

We showed that TT, TU, or both combined, when given soon after the provoking nerve injury, improved 2 laboratory measures of neuropathic pain; namely, they increased both the mechanical withdrawal threshold (indicating less allodynia) and the thermal withdrawal latency (indicating reduced hyperalgesia). Furthermore, these improved behavioral indexes were associated with suppressed levels of spinal IL-6 and Iba1 and increased levels of IL-10. The best results were obtained with the combined use of TT and TU.

The current study and our previous study[7] showed that TT attenuated the mechanical and heat hypersensitivities in a rat model of CCI. This result is similar to the previous reports that exercise decreased pain symptoms in humans[35–37] and diminished postoperative pain[16,17] or neuropathic pain[7,15] in rodents. Assuredly, we demonstrated that TU reduced peripheral neuropathic pain evoked by CCI of the sciatic nerve. Likewise, the current study resembles our previous study in that TU application resulted in both anti-hyperalgesic and anti-allodynic effects in rats after peripheral nerve injury,[38] and either of these effects of TU may be associated with neurokinin-1 receptor (NK1R) expression, substance P, and proinflammatory cytokines.[38] A similar effect was that the use of TU with a frequency of 1 MHz and a maximum intensity of 0.5 W/cm^2 recovered up to 90% of sciatic function in mice with sciatic nerve injury induced by crushing the sciatic nerve.[11] In the present study, the degree of reduction in mechanical allodynia (<30%) and heat hyperalgesia (<65%) by TU or TT was still small and indicated the presence of neuropathic pain, whereas combined TU and TT exhibited a complete reversal of neuropathic pain.

Thermal stimuli are conducted by Aδ- and C-fibers.[39] The corresponding transduction receptors are the A- and C-fiber mechanoheat nociceptors, which respond to thermal and mechanical stimuli.[39] Mechanical allodynia evoked by a light touch is generally accepted to be mediated by low-threshold Aβ fibers in most instances,[39] whereas mechanical

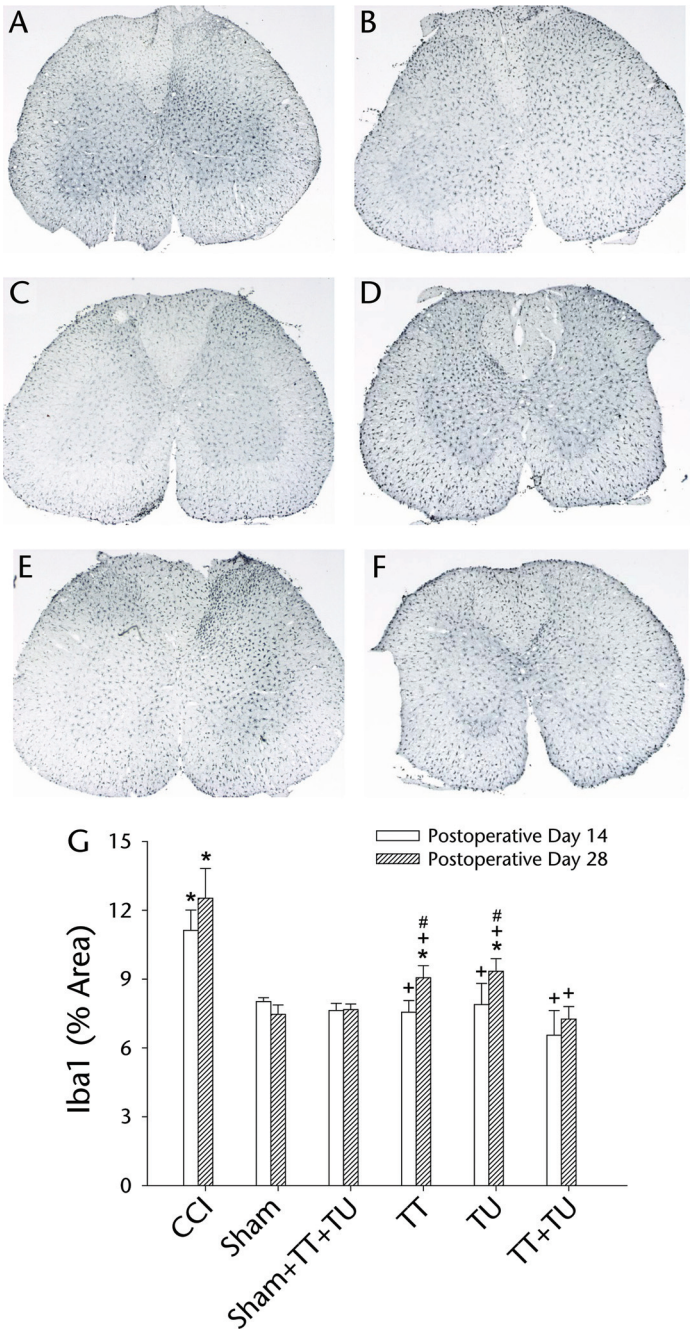

Figure 3.
Immunohistochemical staining of rat spinal cord stained with the anti-Iba1 antibody on day 28 after CCI (A–F) and quantification of ionized calcium-binding adaptor molecule 1 (Iba1) immunoreactivity on days 14 and 28 after CCI (G) in the ipsilateral dorsal horn of the spinal cord in (A) CCI, (B) sham, (C) sham+TT+TU, (D) TT, (E) TU, and (F) TT+TU rats, where CCI=chronic constriction injury, sham=sham operated, sham+TT+TU=sham operated rats that received treadmill training and therapeutic ultrasound, TT=CCI rats that underwent treadmill training, TU=CCI rats that received therapeutic ultrasound, and TT+TU=CCI rats that received treadmill training and therapeutic ultrasound. The data are expressed as the mean area (±standard error of the mean) for 6 rats per group. All images were taken at magnification 2.5× prior to reproduction unless noted. Scale bars: 200 μm. $^{*}P<.05$ compared with the sham group, $^{+}P<.05$ compared with the CCI group, $^{\#}P<.05$ when the TT+TU group was compared with the TU or TT group (1-way analysis of variance followed by post hoc Bonferroni test).

allodynia disappeared by selectively blocking A-fiber input in patients with nerve injury.[40] The fact that thermal and mechanical sensitivity appeared to be differentially influenced by TU and TT raises very interesting questions regarding the specific types of sensory fibers affected by each.

Central or peripheral nerve injury contributes to neuropathic pain.[1] Although the pathogenesis of neuropathic pain involves several neuroplastic mechanisms, central and peripheral sensitization of nociceptive pathways play an important role in the initiation and maintenance of neuropathic pain.[1,2] Despite distinct neuronal and synaptic mechanisms, the proinflammatory cytokines indeed adjust the central sensitization through suppressing the inhibitory neurotransmission and enhancing the excitatory neurotransmission.[41] To address the mechanisms of TU, TT, and both on CCI-evoked neuropathic pain, we focused on the possible role played by IL-6 in the spinal cord. It has been shown that a neuropoietic cytokine (ie, IL-6) can be significantly upregulated in the spinal cord following the peripheral nerve injury.[42–45] In the same way, our study also demonstrated an upregulation of spinal IL-6 in the rats following CCI surgery. Although we cannot be sure whether the mechanisms of proinflammatory cytokines are involved, it is generally believed that proinflammatory cytokine causes pain behavior after intrathecal injection.[41] Furthermore, spinal injection of IL-6-neutralizing antibody markedly postponed the onset of pain.[46]

There was bilateral rather than ipsilateral sensitization. Classic and recent spinal nerve ligation studies[47,48] have shown that inflammatory markers are predominantly increased on the side of injury (ipsilateral) compared with the uninjured (contralateral) side, although there is some evidence that bilateral effects may be observed at very late time points.[49] The current study investigated both sides of the spinal cord. Our results showed that TU, TT, and both suppressed the upregulation of spinal IL-6 in the CCI rats. Among them, the combination of TU and TT exhibited the best effects. This is similar to our previous

finding that TT ameliorated the proinflammatory cytokines in the sciatic nerve of the CCI rats.[7]

In the present study, we showed that the CCI rats exhibited low spinal IL-10 levels, whereas the CCI rats receiving TU, TT, or the combination of both had higher spinal IL-10 levels compared with the sham-operated rats. Our results demonstrated a reduction of IL-10 protein early following peripheral nerve injury,[50] and other proteins showed a significant increase in IL-10 messenger RNA later (35 days) during peripheral nerve injury,[51] which indicates a critical role of the cytokine in nerve regeneration rather than in the early phase of nerve degeneration. In addition, Milligan et al[52] showed that spinal gene therapy elicits an elevation of the IL-10 expression in the spinal cord of naive rodents and attenuates CCI-evoked neuropathic pain. Furthermore, IL-10 suppressed tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) levels or generated free radical oxygen products and increased interferon gamma (IFN-γ) contents by inhibiting the interleukin-2 (IL-2) production of the antigen-presenting cells.[53,54] From this research, IL-10 appears to stand out as a potential candidate for immunotherapy, anti-inflammatory, or immunosuppressive effect and prevents the onset, recurrence, and progression of neuropathy. Therapeutic ultrasound, TT, or the combined therapies increased IL-10 and decreased IL-6 content, suggesting a newly diagnosed strategy for the treatment of peripheral nerve injuries.

One mechanism addressed in the previous studies showed the glial cell activation and subsequent release of certain cytokines, as has been involved in neuropathic pain and the genesis of inflammatory states.[55–59] A robust increased level of Iba1 expression, as a microglial marker, was reported in the spinal cord after peripheral nerve injury.[60] Spinal microglia can rapidly respond to the peripheral nervous system damage because of the wide range of expression of the membrane receptor proteins implicated in a revelation of pathogen-associated or injury-associated molecular patterns.[61] Thus, the results could support our discoveries that a significant increase in Iba1 expression appeared in the spinal cord of the CCI rat with marked pain hypersensitivity.

Recently, it was shown that exercised rats that were given monosodium glutamate had an increased immune-labeled area and decreased Iba1 immunoreactivity intensity in the cerebral cortex.[62] This result showed that the effect could last after 21 days of training.[62] Additionally, CFA-induced synovitis exhibited increased Iba1 staining, whereas low-intensity ultrasound significantly reduced excess Iba1 staining.[63] In the present study, TU, TT, or both lowered the upregulation of Iba1 in the spinal cord. We presume that the treatments (TU, TT, or both) could suppress central sensitization, evoked through nerve injury or inflammation, by reduction of Iba1 expression.

There are limitations to this study. First, we did not have any evidence that so many applications of this halogenated ether compound could not have contributed to the "beneficial" effects ascribed to the ultrasound. In other words, isoflurane does more than just produce sedation.[64] Isoflurane also affects different receptors associated with nociceptive processing.[64] It is unlikely that these antinociceptive benefits continue even after the animals regain consciousness. In this study, serial anesthesia (ie, isoflurane) was used during TU and intervention while animals in groups 2 and 4 were not lightly anesthetized with isoflurane for the same time period and intervals, as the groups receiving TU were noted as the limitations. The sham CCI-treated rats with TU (including isoflurane)+TT (group 3) showed the least change in paw withdrawal thresholds and latencies from baseline. Second, we did not observe that the group 1 animals (the worst-case scenario group) exhibited any "painful" behaviors not observed in the other groups while simply in their cages. These animals actually might be experiencing pain when not being manipulated. Therefore, spontaneous pain evaluation by conditioned place preference should be examined in the future. Third, we did not include the control group of animals that received the ultrasound turned off, indicating that little anesthesia plus ultrasound device massage rules out the potential effects observed in this study. However, our previous study demonstrated that when only the massage was applied with the ultrasound turned off, it did not have potential effects on behavioral measures.[38] Moreover, the usage of the TU device while it was turned off did not change the withdrawal latency, which presumes that the antinociceptive effect by TU occurs on a biophysical basis.[65] In this case, no purely massage-derived effect or placebo was derived.

We concluded that the treatments, regardless of whether alone or in combination, reduced the animals' pain. The co-administration of TT and TU showed the best improvement in neuropathic pain among the 3 therapeutic methods. These results will enrich our understanding for treating pain by controlling Iba1 and inflammatory cytokine expression.

Professor Hung, Ms Huang, and Professor Chen provided concept/idea/research design. Professor Chen provided writing. Ms Huang and Dr Tzeng provided data collection. Dr Tzeng and Professor Wang provided data analysis. Professor Hung and Professor Chen provided project management. Professor Hung, Dr Tzeng, Professor Wang, and Professor Chen provided facilities/equipment. Ms Huang and Professor Wang provided institutional liaisons. Professor Wang and Professor Chen provided administrative support. All authors provided consultation (including review of manuscript before submission).

The experimental protocols were approved by the Experimental Animal Committee of National Cheng Kung University in Taiwan.

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