中國醫藥大學
專題研究計畫成果報告

計畫名稱：經皮神經電刺激合併右啡烷治療大鼠單一神經損傷之療效

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主持人：陳郁文

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目錄

一、前言------------------------------------------------------------- P 2
二、研究方法 -------------------------------------------------------- P 2
三、結果------------------------------------------------------------- P 4
四、討論（含結論與建議）------------------------------------------ P 6
五、參考文獻--------------------------------------------------------- P 6
六、附件二----------------------------------------------------------- P 8
一、前言

Chronic constriction injury of the sciatic nerve may lead to neuropathic pain, which is characterized by hyperalgesia, allodynia, and painful spontaneous burning sensations (Kuphal et al., 2007). Neuropathic pain, which affects large numbers of people worldwide, has become one of the most notable health problems. It hinders the ability of patients to work, walk and sleep, and even their quality of life (Kuphal et al., 2007). Although many available pharmacotherapies (such as antidepressants, antiepileptics) are effective for neuropathic pain, these drugs have incomplete efficacy in neuropathic pain and dose-limiting adverse effects (Saarto and Wiffen, 2005). Therefore, the establishment of new therapeutical approaches for neuropathic pain has been an important field of research in recent years.

It has been known that transcutaneous electrical nerve stimulation (TENS) is an easy to use non-invasive analgesic intervention, applied for diverse pain states and introduced in the early 1970s (Oosterhof et al., 2008). Several studies indicated that transcutaneous electrical nerve stimulation treats for postherpetic neuralgia, spinal cord injury, and neuropathic pain (Barbarisi et al., 2010; Norrbrink, 2009; Somers and Clemente, 2006). In addition, dextrorphan and MK-801, two noncompetitive NMDA receptor antagonists, had antagonistic effects on both the mechanical and thermal allodynia from peripheral nerve injury in rats (Kim et al., 1997; Tal and Bennett, 1993). In addition, substance P (SP) is a pain-related neuropeptide contained in small size DRG neurons. SP immunoreactivity appeared in a subpopulation of medium- to large-sized DRG neurons, and SP induced by peripheral nerve injury (Noguchi et al., 1995).

研究目的

The aim of this study was to examine whether the co-administration of TENS and dextrorphan treats mononeuropathy in the chronic constriction injury model of the rat.

本研究為 8 個月期計畫，分為兩部份：第一部份為利用 TENS 與 dextrorphan 合併治療大鼠神經病變，第二部份為運動訓練治療大鼠神經病變 (此部分已發表於國際期刊(SCI) Anesth Analg 2012，且於致謝中載明計畫名稱，附錄二)。


二、研究方法

2.1. Animals

Male Sprague-Dawley rats weighting 250-300 g from the National Laboratory Animal Center (Taipei, Taiwan) were housed in a climate-controlled room maintained at 21°C with approximately 50% relative humidity in the Animal Center of China Medical University. Lighting was on a 12-hour light-dark cycle (light on at 6:00 a.m.) and food and water were available ad libitum up to the time of testing.

2.2. Groups and design

Animals were divided into five groups: (1) control (CL), (2) chronic constriction
injury (CCI), (3) CCI + TENS (CCIT), (4) CCI + dextrorphan (CCID), and (5) CCI + TENS + dextrorphan (CCITD).

2.3. Surgery

Rats were anesthetized with pentobarbital sodium (45 mg/kg, i.p.). The skin on the lateral surface of the thigh above the greater trochanter of the femur bone was incised via a knife. The sciatic nerve was exposed, and four ligatures (4/0 chromic gut) were tied loosely around the sciatic nerve as described by Bennett and Xie (1988). Loose ligations were tied around the sciatic nerve with 4 chromic gut using 1-mm interligation spacing (Bennett and Xie, 1988). The length of the sciatic nerve thus affected by ligation was 5-6 mm. The degree of constriction of the sciatic nerve was controlled by ligation and sometimes a brief twitch in the muscle surrounding the exposed sciatic nerve was produced. The incision of the muscle, adjacent fascia and skin was closed with 3/0 nylon (Bennett and Xie, 1988), and then the animal with skin wound clips was returned to its cage for recovery. Sham operations involved exposure of the sciatic nerve and its branches with the same procedures but without creating any ligation.

2.4. TENS intervention

TENS was applied to rats through self-adhesive surface electrodes (SoftTouch 9,000; Empi Inc., St. Paul, MN) using an Empi Epix XL transcutaneous electrical nerve stimulator. The stimulator was set to run continuously using no preprogrammed options. The Epix XL uses an asymmetric, bisourced, biphasic waveform with a pulse duration that varies (0 to 400 µs) with intensity. For high-frequency TENS, surface electrode placement was on the chemically denuded, presumably uninvolved skin overlying the left (contralateral to nerve injury in CCI rats) paraspinal usculature. Electrodes were cut to 45 mm (length) by 5 mm (width). The intensity of stimulation was 80% of that needed to produce a visible muscle contraction (30-40 microamperes delivered through 45 mm × 5-mm electrodes) (Somers and Clemente, 2006; Somers and Somers, 1999).

2.5. The treatment of dextrorphan

Dextrorphan (DX) tartrate was purchased from Sigma (St. Louis, MO) and dissolved in 0.9% NaCl (saline) before drug injection. DX (60 mg/kg, i.p.) was injected daily after CCI-treatment. The possible anti-nociceptive effect of DX was examined on postsurgery days 1, 3, 7, and 14 for the behavioral tests.

2.6. Measurement of thermal withdrawal latency and mechanical withdrawal threshold

The animals were tested for thermal hyperalgesia and mechanical allodynia after a period of at least 3 days of habituation to the testing environment. Unless otherwise specified, behavioral tests were conducted before surgery and then evaluated after surgery on days 1, 3, 7, and 14. Thermal hyperalgesia was tested according to the Hargreaves’ Method (Hargreaves et al., 1988).

The lateral plantar surface of the rat was exposed to a beam of radiant heat through a Plantar Analgesia Meter (IITC Life Science Instruments, Woodland Hills, CA) was used to measure the response. Briefly, rats were placed individually in a clear plexiglass chamber (22 cm [length] x 22 cm [width] x 13.3 cm [height]), and the animals stood on a glass sheet with the temperature maintained at 30 ±1°C to decrease the influence of the temperature in different seasons. The paw withdrawal latency was recorded. The heat stimulation was repeated 3 times at intervals of 5 minutes for each test and the mean was calculated. A maximal automatic cut-off latency of 20 seconds was used to prevent tissue damage.

For assessment of mechanical allodynia, rats were placed individually in a clear
plexiglass chamber (22 cm [length] x 22 cm [width] x 13.3 cm [height]) and supported by a wire mesh floor (40 cm [width] x 50 cm [length]). An electronic von Frey filament Analgesia Meter (IITC Life Science Instruments, Woodland Hills, CA) was applied at the lateral plantar surface of the rat. The paw withdrawal threshold was recorded. The mechanical stimulation was repeated 3 times at intervals of 5 minutes for each test and the mean was calculated.

2.7. Substance P analysis

Protein samples (30 μg/lane) were separated using 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) at a constant voltage of 75 V. (Chen et al., 2007) Electrophoresed proteins were transferred to a polyvinylidene difluoride (PVDF) membrane with a 0.45 μm pore size (Millipore, Bedford, MA,) using a transfer apparatus (Bio-Rad, Hercules, CA, USA). The PVDF membrane was incubated in 5% milk in TBS buffer. The membrane was blocked in TBS (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20, pH 7.5) containing 5% skim milk (Difco, Detroit, MI) for 1 hour. Rat monoclonal anti-substance P primary antibody (250865, San Diego, CA 92126) was diluted to 1:1000 in antibody binding buffer overnight at 4°C. The membrane was then washed three times with TBS (10 minutes per wash) and incubated for 1 hour with horseradish peroxidase-conjugated AffiniPure goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and diluted 5,000-fold in TBS buffer at 4°C. The membrane was washed in TBS buffer for 10 minutes three times. Immunodetection for substance P was performed using the enhanced chemiluminescence ECL Western blotting luminal reagent (Santa Cruz Biotechnology) and then the membrane was quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Tokyo, Japan).

2.8. Statistic analysis

Data are means ± S.E. The analysis was done using one-way ANOVA for post-hoc analysis to compare the tactile alldynia, thermal hyperalgesia, and substance P expression in each group. Statistical significance was set at $P < 0.05$.

三、結果

第一部分結果:

3.1. Thermal hyperalgesia

The rats developed a significant decrease in thermal withdrawal latency on day 1, 3, and 7 after the sciatic nerve injury when compared to rats on day 0 in CCI group in Figure 1. On day 3 after CCI, rats after treatment at 0.5 and 1 hours in CCIT, CCITD or CCID groups showed the increasing thermal withdrawal latency when compared with the condition of rats before treatment in Figure 2. On day 1, 3, and 7 after CCI, thermal withdrawal latency increased significantly in the CCIT, CCITD or CCID group, compared with the CCI group in Figure 1.

3.2. Substance P expression

The substance P expression was significantly higher in the dorsal root ganglion of CCI-group rats on day 3 after CCI (Fig. 3). Three days after dextorphan or TENS treatment, substance P expression was significantly less in the dorsal root ganglion of CCID-, CCITD- or CCID-group than CCI-group rats in Figure 3.
Fig. 1. The time course of thermal withdrawal latency in CL (n=3), CCI (n=4), CCIT (n=3), CCITD (n=5), and CCID (n=3) rats (CL: control; CCI: chronic constriction injury; CCIT: CCI + TENS [transcutaneous electrical nerve stimulator]; CCID: CCI + dextrorphan; CCITD: CCI + TENS [transcutaneous electrical nerve stimulator] + dextrorphan). Data are presented as mean ± SEM.

Fig. 2. The time course of thermal withdrawal latency in CCIT (n=5), CCID (n=5) and CCITD (n=5) rats (CCIT: chronic constriction injury + TENS [transcutaneous electrical nerve stimulator]; CCID: CCI + dextrorphan; CCITD: CCI + TENS + dextrorphan). Data are presented as mean ± SEM.
Fig. 3. The levels of substance P in dorsal root ganglion on day 3 after CCI in different groups of rats: CL, CCI, CCID and CCITD (CL: control; CCI: chronic constriction injury; CCID: CCI + dextrorphan; CCITD: CCI + TENS [transcutaneous electrical nerve stimulator] + dextrorphan). The values are presented as mean ± SEM for 4 rats per group.

第二部份結果：為運動訓練治療大鼠神經病變 (此部分已發表於國際期刊(SCI) Anesth Analg 2012, 且於致謝中載明計畫名稱, 附件二)。

四、討論 (含結論與建議)
根据此計畫的結果，我們發現 dextrorphan 與 TENS 或運動可以減緩改善神經病變導致的痛覺過度敏感(thermal hyperalgesia)，同時神經組織亦見較少的 substance P 表現量，其它相關結果詳見附件二。感謝國科會提供經費，以致於有機會執行此大專生研究計畫。

註：計畫中第二部分 (附件二) 已發表於國際期刊(SCI) Anesth Analg 2012。

五、參考文獻
Kim, Y.I., Na, H.S., Yoon, Y.W., Han, H.C., Ko, K.H., Hong, S.K., 1997. NMDA receptors are important for both mechanical and thermal allodynia from peripheral nerve injury in rats. Neuroreport 8, 2149-2153.
Exercise Training Attenuates Neuropathic Pain and Cytokine Expression After Chronic Constriction Injury of Rat Sciatic Nerve

Yu-Wen Chen, PhD,* Yung-Tsung Li, MS,* Yu Chung Chen, MS,† Zong-Ying Li, BS,* and Ching-Hsia Hung, PhD‡

BACKGROUND: The underlying mechanism of exercise on neuropathic pain is not well understood. We investigated whether physical exercise regulates the functional recovery and heat shock protein 72 (Hsp72), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) expression after chronic constriction injury (CCI) of the sciatic nerve.

METHODS: Male Sprague–Dawley rats were divided into 7 groups: control, sham operated (SO), SO with swimming or treadmill exercise (SOSE or SOTE), CCI, CCI with swimming or treadmill exercise (CCISE or CCITE). We recorded body weight, thermal withdrawal latency, and mechanical withdrawal threshold as well as Hsp72, TNF-α, and IL-1β expression in sciatic nerve.

RESULTS: The body weights in the control and SO groups were heavier than those in the SOSE, SOTE, CCI, CCISE, and CCITE groups. CCI rats with swimming or treadmill exercise showed significant increase in thermal withdrawal latency and mechanical withdrawal threshold when compared with CCI rats without exercise on day 21 after CCI. Both CCISE and CCITE groups demonstrated greater Hsp72 expression and lower TNF-α or IL-1β level than did the CCI group in sciatic nerve on day 21 after CCI.

CONCLUSIONS: These results suggest that progressive exercise training decreases peripheral neuropathic pain as well as TNF-α and IL-1β overproduction and increases HSP72 expression after CCI of the sciatic nerve. (Anesth Analg 2012;X:●●●●●●)

Neuropathic pain hinders the ability of patients to work, walk, and sleep, and even their quality of life. Clinically, patients with neuropathic pain after nerve injury often complain of continuing burning pain as well as pain to light touch. Although many available pharmacotherapies (such as antidepressants, antiepileptics) are effective for neuropathic pain, these drugs produce side effects. We wondered whether nonpharmacotherapies might be treatment options for neuropathic pain.

There is a growing body of evidence that exercise decreases symptoms of acute pain in humans has numerous beneficial effects on chronic diseases, has an anti-inflammatory effect, and reduces neuropathic pain in rodents. Neuropathic pain provokes varying degrees of local inflammatory responses and overexpression of inflammatory cytokines in locally activated macrophages, Schwann cells, and other glial cells. Furthermore, studies have shown that proinflammatory cytokines (e.g., tumor necrosis factor-α [TNF α] interleukin-1β [IL-1β]) induce pain and that treatment with anti-inflammatory cytokines or inhibitors of proinflammatory cytokines relieve pain. In addition, it has been presumed that exercise pretraining can induce heat shock protein 72 (Hsp72) expression in multiple organs and protect against cerebral ischemia and damage to cerebral neurons in rats. However, only a few studies have examined the effects of swimming and treadmill exercise on neuropathic pain, cytokines, and Hsp72.

The aim of this study was to investigate whether exercise training (swimming or treadmill exercise), a nonpharmacotherapy, reduces peripheral neuropathic pain and expression of proinflammatory cytokines and increases Hsp72 expression after chronic constriction injury (CCI) of the sciatic nerve in rats. Hyperalgesia, allodynia, TNF-α, IL-1β, and Hsp72 were evaluated in CCI rats with and without treadmill or swimming exercise.

METHODS

Animal Preparation

Experiments were performed on 250- to 300-g male Sprague–Dawley rats (National Laboratory Animal Center, Taipei, Taiwan). The rats were housed in a climate-controlled room maintained at 21°C with approximately 50% relative humidity in the Animal Center of China Medical University. Lighting was on a 12-hour light–dark cycle (light on at 8:00 AM), and food and water were available ad libitum up to the time of testing. The experimental protocols to perform this study were approved by the Institutional Animal Care and Use Committee of China Medical University, Taiwan. Effort was made to minimize discomfort of the animals and reduce the number of
Experimental animals. All studies were conducted according to International Association for the Study of Pain ethical guidelines.19

Groups and Design
Rats were divided into 7 groups: (1) normal rats (control), (2) rats with CCI, (3) rats with CCI combined with swimming exercise treatment (CCISE), (4) rats with CCI combined with treadmill exercise treatment (CCITE), (5) rats sham operated (SO), (6) rats sham operated combined with swimming exercise treatment (SOSE), (7) rats sham operated combined with treadmill exercise treatment (SOTE). Some rats were considered for the overall behavioral analysis and body weight \((n = 20, 20, 10, 10, 20, 10, 10\) for 2 control, 2 SO, SOTE, SOSE, CCITE, 2 CCI, and CCISE, respectively), while some rats were killed for tissue analysis (TNF-\(\alpha\) on day 21 after CCI \((n = 5, 5, 5\) for CCITE, CCI, and CCISE, respectively), some rats were killed for IL-1beta analysis on day 21 after CCI \((n = 5, 5, 5\) for CCITE, CCI, and CCISE, respectively), and other rats were killed for Hsp72 analysis on day 21 after CCI \((n = 5, 5, 5\) for CCITE, CCI, and CCISE, respectively). Experimenters were blinded for different experimental group assignment. The rats rested 1 day after the operation until the start of training.

Surgery
The animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). The skin on the lateral surface of the thigh was incised, and an incision was made through the skin, freeing tissue adhesions along about 7 mm of the sciatic nerve just distal to the greater trochanter of the femur bone. The sciatic nerve was exposed through the biceps femoris muscle, and then its 3 terminal branches (the sural, common peroneal, and tibia nerves) were identified. Four ligatures (4/0 black silk) were tied loosely around the sciatic nerve as described by Bennett and Xie.20 The left sciatic nerve was exposed proximal to the sciatic trifurcation, and an approximately 7-mm section of the nerve was freed from the surrounding tissue. Great care was taken to tie the ligatures so that the diameter of the nerve was seen to be just barely constricted. The length of nerve thus affected by ligation was 5 to 6 mm. The incisions of the muscle, adjacent fascia, and skin were closed with 4/0 silk20 and then with wound clips, and the animal was returned to its cage for recovery. Sham operations involved exposure of the sciatic nerve and its branches with the same procedures but without creating any lesion.

Swimming Exercise
The protocol for swimming exercise training1 is presented in Table 1. Rats were placed in a plastic container (74 cm [length] \(\times\) 53 cm [width] \(\times\) 60 cm [height]) holding approximately 43 cm of water (37°C \(\pm\) 0.5°C). In rare instances, animals needed to be mildly stimulated to swim by nudging the nape of their neck with a pen. This protocol ensured a full session of exercise conditioning. After each exercise session, animals were gently dried with a cloth towel.

<table>
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<th>Table 1. Graded Swimming Exercise Protocols</th>
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<td>Exercise period (minutes)</td>
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<td>7–39</td>
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All swimming animals adapted to the water depth and temperature 2 days before surgery. *According to the swimming protocol of Kuphal et al.1

<table>
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<th>Table 2. Graded Treadmill Exercise Protocols</th>
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<td>Exercise rate (km/h)</td>
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The rats ran on a treadmill 5 days per week for 6 weeks. On the first week all rats were acclimated to the track and ran for 15 minutes at 1.2 km/h, 0% slope, for 3 days. The duration and intensity of the exercise were increased progressively.

Treadmill Exercise
The protocol for treadmill exercise training was performed according to previously described methods.18,21 The protocol is presented in Table 2. In brief, rats were trained to run on a treadmill (Chanson, CS-5515, Taipei, Taiwan) 5 days a week for 6 weeks. Initially, the rats were acclimated to the training program and ran for 15 minutes at 1.2 km/hr, 0% slope, for 3 days. They ran without electrical stimulation. The duration and intensity of the exercise were increased progressively so that the animals were running for 30 minutes at 1.2 km/hr, 30 minutes at 1.8 km/hr and 60 minutes at 1.8 km/hr after 1, 2, and 3 weeks of training, respectively. The work rate of rats on this training protocol was about 70%–75% of their maximal oxygen consumption. If the rats’ feet were hurt during the training protocol, they were withdrawn from the study.

Thermal and Mechanical Sensitivity
We interpret the decrease in heat latency and mechanical threshold as hyperalgesia and allodynia, respectively. The rats were tested for thermal hyperalgesia and mechanical allodynia after a period of at least 3 days of habitation to the testing environment. Unless otherwise specified, behavioral tests were conducted 1 day before surgery, the day of surgery, and on days 1, 3, 7, 14, 21, 28, 35, and 39. All measurements were performed between 9:00 AM and 11:00 AM. For consistency, 1 experienced investigator (Dr. Chen) who was blinded to the groups was responsible for behavioral tests.

Thermal hyperalgesia was tested according to the Hargreaves’ method.22 Briefly, rats were placed individually in a
calculated. A maximal automatic cutoff latency of 20 sec-
tions of 5 minutes for each test, and the mean was
calculated. A maximal automatic cutoff latency of 20 sec-
onds was used to prevent tissue damage.

For assessment of mechanical allodynia, rats were
placed individually in a clear plexiglass chamber (22 cm
[length] × 22 cm [width] × 13.3 cm [height]) and supported
by a wire mesh floor (40 cm [width] × 50 cm [length]). An
electronic von Frey filament analgesia meter (IITC Life
Science Instruments, Woodland Hills, CA) was applied at
the lateral plantar surface of the left hindpaw. The paw
withdrawal threshold was recorded. The withdrawal re-
sponses evoked by thermal stimulation were determined,
including foot lifting, shaking, licking, and squeaking. Paw
movements associated with weight shifting or locomotion
were not counted. Heat stimulation was repeated 3 times
at intervals of 5 minutes for each test, and the mean was
calculated. A maximal automatic cutoff latency of 20 sec-
tions of 5 minutes for each test, and the mean was
calculated.

Tissue Preparation
The rats were anesthetized with urethane (1.67 g/kg, i.p.)
and killed on day 21 after CCI. Under aseptic conditions,
skin was cut to expose the left sciatic nerve, proximal to the
trifurcation (about 1 cm), before the 4 ligatures were
removed. The nerve specimen was immediately stored at
−80°C for protein assay.

Ice cold (4°C) homogenization buffer was added (300
µL/each sciatic nerve). The homogenization buffer was
freshly prepared by adding protease inhibitor (P 8340
cocktail, Sigma–Aldrich, St. Louis, MO) to T-PER™ Tissue
Protein Extraction Reagent (Pierce Chemical Co., Rockford,
IL) before tissue lysis. After adding the buffer, a homoge-
nization probe (Tissue Tearor, Polytron; Biospec Products,
Inc., Bartlesville, OK) was applied for 20 seconds on ice
at 21,000 revolutions per minute (rpm). The homogenized
samples were then centrifuged for 40 minutes at a speed of
13,000 rpm at 4°C, stored at

The change in body weight after surgery is shown in Figure
1B and CCISE groups) in Figure 1B showed significant main
effects for groups (F\(_{4,45} = 22.13, P < 0.0001\); F\(_{4,45} = 20.34,
P < 0.0001\), time (F\(_{9,405} = 10.31, P < 0.0001\); F\(_{9,405} = 8.77,
P < 0.0001\), and significant interaction (F\(_{36,405} = 5.89, P <
0.0001\); F\(_{36,405} = 4.72, P < 0.0001\), respectively. Post hoc

RESULTS
Body Weight
The change in body weight after surgery is shown in Figure
1. ANOVA for repeated measures (including control, SO,
SOTE, CCI, and CCITE groups) in Figure 1A and ANOVA
for repeated measures (including control, SO, SOSE, CCI,
and CCISE groups) in Figure 1B showed significant main
effects for groups (F\(_{4,45} = 22.13, P < 0.0001\); F\(_{4,45} = 20.34,
P < 0.0001\), time (F\(_{9,405} = 10.31, P < 0.0001\); F\(_{9,405} = 8.77,
P < 0.0001\), and significant interaction (F\(_{36,405} = 5.89, P <
0.0001\); F\(_{36,405} = 4.72, P < 0.0001\), respectively. Post hoc

Cytokine Evaluation
The concentrations of TNF-α and IL-1β in the supernatants
were determined using the DuoSet® ELISA Development
Kit (R&D Systems, Minneapolis, MN). All experimental
procedures were performed in accordance with the instruc-
tions. Plates were individually inserted into the plate
reader for reading optical density using a 450-nm filter.
Data were then analyzed using Ascent Software for iEMS
Reader and a 4-parameter logistics curve fit. Data were
expressed in pg/mg protein of duplicate samples.

Hsp72 Analysis
Protein samples (30 µg/lane) were separated using 12%
SDS polyacrylamide gel electrophoresis (SDS-PAGE) at
a constant voltage of 75 V. \(^18\) Electrophoresed proteins were
transferred to a polyvinylidene difluoride (PVDF) mem-
brane with a 0.45-µm pore size (Millipore, Bedford, MA)
using a transfer apparatus (Bio-Rad, Hercules, CA). The
PVDF membrane was incubated in 5% milk in tris buffered
saline (TBS) buffer. The membrane was blocked in TBS (20
mM Tris, 500 mM NaCl, and 0.1% Tween 20, pH 7.5)
containing 5% skim milk (Difco, Detroit, MI) for 1 hour.
Mouse monoclonal antiHsp72 primary antibody (SPA 810;
StressGen Biotechnologies, Victoria, British Columbia,
Canada) was diluted to 1:500 in antibody binding buffer
overnight at 4°C. The membrane was then washed 3 times
with TBS (10 minutes per wash) and incubated for 1 hour
with horseradish peroxidase-conjugated goat antimouse
secondary antibody (Santa Cruz Biotechnology, Santa
Cruz, CA) and diluted 500-fold in TBS buffer at 4°C. The
membrane was washed in TBS buffer for 10 minutes 3
times.

Immunodetection for Hsp72 was performed using the
enhanced chemiluminescence ECL Western blotting lumili-
neal reagent (Santa Cruz Biotechnology), and then the
membrane was quantified using a Fujifilm LAS-3000
chemiluminescence detection system (Tokyo, Japan).

Statistical Analysis
The results are presented as mean ± SEM. The differences
among data related to body weight (Fig. 1), paw with-
drawal latency (Figs. 2 and 3), and paw withdrawal thresh-
old (Figs. 2 and 3) were analyzed by 2-way analysis of
variance (ANOVA) of repeated measures, followed by
Tukey–Kramer post hoc comparison. Exercise treatment
was the between-subjects factor, and time was the repeated
measure. The differences in TNF-α (Fig. 4A), IL-1β (Fig.
4B), and Hsp72 (Fig. 5) were determined using 1-way
ANOVA followed by post hoc Tukey test for multiple
comparisons. Statistical calculations were performed using
SPSS for Windows (version 17.0, SPSS Inc., Chicago, IL).
Differences were considered significant at P < 0.05.
comparisons demonstrated significant differences between control (or SO) and SOTE (or CCI, CCITE) groups (P/H11021 0.05, Tukey–Kramer, Fig. 1A) and between control (or SO) and SOSE (or CCI, CCISE) groups (P/H11021 0.05, Tukey–Kramer, Fig. 1B), respectively. There was no significant difference between the SO and control groups (Fig. 1A and 1B). The CCISE (Fig. 1B) and CCITE (Fig. 1A) groups had no significant change in body weight when compared with the CCI group, respectively. Furthermore, the animals’ grooming, sleep–wake cycles, and social interaction in the cage were not obviously affected (data not shown).

**Thermal and Mechanical Sensitivity**

ANOVA of repeated measures (including control, SO, SOTE, SOSE, CCITE, CCI, and CCISE groups) in Figure 2A and ANOVA of repeated measures (including control, SO, SOTE, CCI, and CCITE groups) in Figure 3A for thermal withdrawal latency demonstrated a significant main effect for groups (F_4,435 = 58.88, P < 0.0001; F_4,435 = 47.39, P < 0.0001), time (F_9405 = 41.58, P < 0.0001; F_9405 = 39.46, P < 0.0001), and significant interaction (F_36,405 = 8.74, P < 0.0001; F_36,405 = 9.56, P < 0.0001), respectively. Post hoc comparisons showed no significant differences among control, SO, and SOSE (or SOTE) for thermal withdrawal latency (P > 0.05, Tukey–Kramer).

In SO and CCI (or CCITE) groups (Fig. 2A), the significant difference in thermal withdrawal latency was maintained from day 3 to day 21 (P < 0.05, Tukey–Kramer). Post hoc comparisons demonstrated significant differences between CCITE and CCI groups from day 3 to day 21 (P < 0.05, Tukey–Kramer). In the CCI group in comparison with SO group (Fig. 3A), the significant difference in thermal withdrawal latency was maintained from day 3 to day 28 (P < 0.05, Tukey–Kramer), while in the CCISE group only
from day 7 to day 14. Post hoc comparisons demonstrated significant differences between CCISE and CCI groups from day 3 to day 21 \((P < 0.05, \text{Tukey–Kramer})\).

ANOVA of repeated measures (including control, SO, SOTE, CCI, and CCITE groups) in Figure 2B and ANOVA of repeated measures (including control, SO, SOSE, CCI, and CCISE groups) in Figure 3B for mechanical withdrawal threshold showed a significant main effect for groups \(F_{4,45} = 66.91, P < 0.0001; F_{4,45} = 66.91, P < 0.0001\), time \(F_{9405} = 51.76, P < 0.0001; F_{9405} = 67.82, P < 0.0001\), and significant interaction \(F_{36,405} = 9.45, P < 0.0001; F_{36,405} = 8.41, P < 0.0001\), respectively. Post hoc comparisons demonstrated no significant differences among control, SO, and SOSE (or SOTE) for mechanical withdrawal threshold \((P > 0.05, \text{Tukey–Kramer})\).

Post hoc comparisons showed significant differences in mechanical withdrawal threshold between CCI and SO groups from day 1 to day 35, between CCITE and SO groups from day 1 to day 28, and between CCITE and CCI groups from day 21 to day 35 (Fig. 2B; \(P < 0.05, \text{Tukey–Kramer}\)). In SO and CCI (or CCISE) groups (Fig. 3B), the significant difference in mechanical withdrawal threshold was maintained from day 1 to day 35 \((P < 0.05, \text{Tukey–Kramer})\). Furthermore, the CCI group was again significantly different from the CCISE group from day 14 to day 21, showing an increase in mechanical withdrawal threshold \((P < 0.05, \text{Tukey–Kramer})\).

Furthermore, on day 21 after CCI, data showed a significant increase in thermal withdrawal latency and mechanical withdrawal threshold in the CCITE or CCISE group when compared with the CCI group. Therefore, we selected day 21 after CCI to evaluate TNF-\(\alpha\), IL-1\(\beta\), and Hsp72 expression in sciatic nerve. There was no significant difference in thermal withdrawal latency and mechanical

Figure 3. Time courses of thermal withdrawal latency (A) and mechanical withdrawal threshold (B) in control, SO, SOSE, CCI, and CCISE rats, where SO = sham operation; SOTE = sham operation with treadmill exercise training; SOSE = sham operation with swimming exercise training; CCI = chronic constriction injury; CCITE = chronic constriction injury with treadmill exercise training; CCISE = chronic constriction injury with swimming exercise training. The paw withdrawal latency (sec) and threshold (g) to heat and mechanical stimulation were not significantly different between the SO or SOSE group in comparison with the control group. Data are presented as mean \(\pm\) SEM for 10 rats per group. The asterisk indicates \(P < 0.05\) when the CCITE group was compared with the CCI group; the plus symbol indicates \(P < 0.05\) when the CCI or CCITE group was compared with the SO group (2-way analysis of variance followed by post hoc Tukey–Kramer test).

Figure 4. The levels of tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) (A) and interleukin (IL)-1\(\beta\) (B) on day 21 after CCI in sciatic nerve in different groups of rats: CCI, CCITE, and CCISE (SO = sham operation; SOTE = sham operation with treadmill exercise training; SOSE = sham operation with swimming exercise training; CCI = chronic constriction injury; CCITE = chronic constriction injury with treadmill exercise training; CCISE = chronic constriction injury with swimming exercise training). The values are presented as mean \(\pm\) SEM for 5 rats per group. Symbols (* and **) indicate \(P < 0.05\) and \(P < 0.01\), respectively, when the CCITE or CCISE group was compared with the CCI group (1-way analysis of variance followed by post hoc Tukey test).
Exercise Decreases Neuropathic Pain

Cytokine Expression

Figure 4A and 4B depict the levels of TNF-α and IL-1β in sciatic nerve of CCI, CCISE, and CCITE rats after exercise training for 21 days (day 21 after CCI). The expression of TNF-α was decreased in the CCITE group (59.4 ± 3.2 pg/mg protein, P < 0.05) or CCISE group (60.9 ± 2.9 pg/mg protein, P < 0.05) in comparison with the CCI group (75.3 ± 2.1 pg/mg protein) on day 21 after CCI (Fig. 4A). The expression of IL-1β was decreased in the CCITE group (69.3 ± 8.8 pg/mg protein, P < 0.01) and CCISE group (92.3 ± 24.0 pg/mg protein, P < 0.05), in comparison with the CCI group (162.9 ± 19.8 pg/mg protein) on day 21 after CCI, as shown in Figure 4B.

Hsp72 Expression

Figure 5 demonstrated the expression of Hsp72 in sciatic nerve after CCI in 3 different groups. The Hsp72 level in sciatic nerve was significantly increased 3.2-fold in the CCITE group (P < 0.01) and 2.1-fold in the CCISE group (P < 0.05) on day 21 after CCI when compared with the CCI group (Fig. 5).

DISCUSSION

The main finding of this study is that swimming and treadmill exercises appeared to retard peripheral neuropathic pain after CCI of the sciatic nerve in rats. After CCI, rats with swimming or treadmill training had decreased TNF-α and IL-1β expression and increased levels of HSP 72 in sciatic nerve when compared with rats after CCI without exercise training.

Effects of Exercise on Thermal and Mechanical Sensitivity

Physical exercise is often recommended to patients who have chronic pain. For instance, it has been shown that treadmill and swimming exercises were found to ameliorate spinal cord injury–induced allodynia and restore normal sensation after spinal cord contusion in rats.23 Bement and Sluka’s study demonstrated that low-intensity exercise reversed mechanical hyperalgesia in a chronic muscle pain rat model through the activation of opioid receptors.24 This study also indicated that the CCI rats in the swimming or treadmill exercise group had attenuated thermal hyperalgesia and mechanical allodynia on day 21 after CCI when compared with the CCI rats not in exercise groups (Figs. 2 and 3). Our results are in agreement with those of previous studies, which reported that swimming exercise attenuated behavioral hypersensitivity in formalin- and nerve injury–induced animal models of persistent pain.1

The more recently published information suggests that intense exercise may exacerbate hyperalgesia25,26; for example, the spread of hyperalgesia was enhanced via fatigue by having mice spontaneously run in a running wheel for 2 hours, but not hyperalgesia at the site of insult.26 It has been shown that moderate-intensity exercise training cannot treat but can significantly decrease deep and cutaneous tissue mechanical hypersensitivity induced via acidic saline injection.27 In our study, the degree of reduction in decreased thermal withdrawal latencies (maximal 30%) and mechanical von Frey thresholds (<50%) by exercise was quite small and demonstrates the relevance of the findings in relation to neuropathic pain that is still present. Our study is consistent with the findings of animal studies, in that exercise does not completely reverse the painful condition.27

In this study, SOSE, SOTE, CCI, CCISE, or CCITE rats showed decreases in body weight in comparison with control or SO rats. We speculate that rats suffered from stress (e.g., CCI) and exercise, and therefore a similar trend of decreasing body weight in the SOSE, SOTE, CCI, CCISE, or CCITE rats was found. Weight reduction is one of the health benefits of regular exercise that should be emphasized and reinforced by every mental health professional to their patients.28

Our previous study demonstrated that progressive exercise training for at least 3 weeks induces Hsp72 overexpression in many vital organs and attenuates overproduction of tissue cytokines, including TNF-α, and arterial hypotension during heatstroke.29 This present study also showed that 3 weeks (21 days) of exercise training decreases heat hyperalgesia and mechanical allodynia in rats after CCI (Figs. 2 and 3). Therefore, we tested sciatic nerve Hsp72, IL-1beta, and TNF-α expression on day 21 after CCI.

Exercise Prevents the Increase in Sciatic TNF-α and IL-1β

The appearance of cytokines in plasma or in the tissues (e.g., nerves, muscles, bones) has been reported in work-related musculoskeletal disorders in humans30 and in animal models.31 However, the relationships among cytokines, pain, and exercises have not been analyzed. Our
results showed that treadmill or swimming exercise training attenuated TNF-α and IL-1β expression on day 21 after CCI. This evidence may provide a reasonable explanation for why exercise training can partly alleviate neuropathic pain after CCI in rats.

Evidence has been presented that neuropathic pain consequent to peripheral nerve injury is associated with local inflammation and overexpression of inflammatory cytokines.32 It is well known that CCI induces axons to become hypersensitive and enhances retrograde transmission to cell bodies in the dorsal root ganglia and spinal cord with subsequent release of some mediators. These mediators were able to activate the microglia cells via specific receptors and induce phosphorylation of p38 mitogen-activated protein kinase in spinal cord, where they may alter gene expression of the neurons.33–35 Moreover, the hyperactive microglia result in the release of proinflammatory substances, including cytokines, prostaglandin E2, and excitatory amino acids (such as glutamate and aspartate) that alter the responses of dorsal horn cells and maintain neuropathic pain states.8,36

Effects of Exercise on Hsp72

Voluntary exercise for 7 days up-regulates the small heat shock protein Hsp27 in the hippocampus,37 and forced long-term exercise in mice has been reported to increase heat-shock protein/cognate 70 (Hsp/C 70).29 Our previous study demonstrated that a 3-week, but not a 1- or 2-week, exercise training regimen conferred significant protection against hyperthermia, decreased cardiac output, arterial hypotension, and increased serum or tissue levels of TNF-α, and improved survival during heatstroke.29 A 3-week exercise training treatment is able to maintain a high level of HSP72 in several vital organs for only 3 to 4 days,29 and a >3-fold overexpression of Hsp72 in the nucleus tractus solitarii may play an important role in protecting against hemodynamic dysfunction during heatstroke onset.14 In this study, we noted that swimming or treadmill exercise retarded mechanical allodynia and heat hyperalgesia and significantly increased Hsp72 expression on day 21 after CCI. Therefore, HSP72 expression in the sciatic nerve after 3 weeks of progressive exercise may play an important role throughout the sciatic nerve injury period.

In agreement with our results, exercise has been shown to be beneficial for an anti-inflammatory effect and neuropathic pain resolution.1,7 Several studies demonstrated that exercise-induced modulation of heat shock factor-1 (HSP’s transcription factor) aggregation subsequently affects expression of Hsp72 in multiple organs or neurons of rats.18,29,37,38 In addition, the effect of treatment with BRX-220 (coinducer of HSPs) on the expression of Hsp70 leads to either slowly developing analgesic actions or enhancement of recovery processes in rats after L5 spinal nerve ligation.39 Moreover, it has been proven that the increase in the expression of HSPs can decrease the production of proinflammatory cytokines.40 Our results revealed that swimming or treadmill exercise training significantly promoted Hsp72 expression and ameliorated the CCI-induced expression of proinflammatory cytokines (TNF-α and IL-1β) in rat sciatic nerve. Furthermore, we also demonstrated that swimming or treadmill exercise training decreases CCI-induced neuropathic pain.

Although we did not provide direct evidence of the mechanism of Hsp72 that attenuated proinflammatory cytokine expression in this study, accumulated evidence shows that HSPs can decrease the production of the proinflammatory cytokines.40 However, we did note that the observations in this study on thermal hyperalgesia, mechanical allodynia, TNF-α, IL-1β, and Hsp72 are, at present, merely coincident.

Our study demonstrated that swimming and treadmill exercises increase HSP 72 expression in sciatic nerve of CCI rats and ameliorate thermal hyperalgesia, mechanical allodynia, and the expressions of TNF-α and IL-1β in sciatic nerve. 

DISCLOSURES

Name: Yu-Wen Chen, PhD.
Contribution: This author helped design the study, conduct the study, and analyze the data.
Attestation: Yu-Wen Chen has seen the original study data, reviewed the analysis of the data, approved the final manuscript, and is the author responsible for archiving the study files.
Name: Yung-Tsung Li, MS.
Contribution: This author helped conduct the study and analyze the data.
Attestation: Yung-Tsung Li has seen the study data, reviewed the analysis of the data, approved the final manuscript, and is the author responsible for archiving the study files.
Name: Yu Chung Chen, MS.
Contribution: This author helped design the study, conduct the study, and analyze the data.
Attestation: Yu Chung Chen has seen the original study data, reviewed the analysis of the data, approved the final manuscript, and is the author responsible for archiving the study files.
Name: Zong-Ying Li, BS.
Contribution: This author helped conduct the study and analyze the data.
Attestation: Zong-Ying Li has seen the original study data, reviewed the analysis of the data, approved the final manuscript, and is the author responsible for archiving the study files.
Name: Ching-Hsia Hung, PhD.
Contribution: This author helped design the study, conduct the study, analyze the data, and write the manuscript.
Attestation: Ching-Hsia Hung has seen the original study data, reviewed the analysis of the data, approved the final manuscript, and is the author responsible for archiving the study files.

This manuscript was handled by: Tony L. Yaksh, PhD.

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Exercise Decreases Neuropathic Pain

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