

# 中國醫藥大學

## 專題研究計畫成果報告

計畫名稱：低能量雷射對於慢性神經病變的運動功能恢復、降低神經痛及促進神經修復之影響及分子生物學機轉

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## **Abstract**

**Objective.** The purposes of this study are (1) to investigate the effect of Low-level laser therapy (LLLT) on accumulation of hypoxia-inducible factor-1 alpha (HIF-1 $\alpha$ ), proinflammatory cytokines tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin-1 $\beta$  (IL-1 $\beta$ ) for controlling neuropathic pain, and (2) to assess the effect of LLLT on activation of vascular endothelial growth factor (VEGF) and nerve growth factor for promoting functional recovery in rat model with chronic constrictive injury (CCI).

**Design:** Experimental animal controlled trial.

**Setting:** Musculoskeletal and neuroscience laboratory in a Medical University, Taiwan.

**Animals:** Adult male Sprague–Dawley rats (N=40) (body weight, 250–300 g).

**Interventions:** CCI was induced by placing four loose ligatures around the sciatic nerve of rats. Treatments of LLLT (660 nm, 9 J/cm<sup>2</sup>) or sham-irradiation (0 J/cm<sup>2</sup>) were performed at CCI sites after 7 days of surgery and given for 7 consecutive days.

**Main Outcome Measures:** Effects of LLLT in CCI animals were determined by measuring mechanical paw withdrawal threshold (MPWT), sciatic, tibial and peroneal function indexes (SFI, TFI and PFI), and histopathological and immunoassay analyses.

**Results.** It was found that LLLT significantly improved MPWT, SFI, TFI and PFI after CCI. LLLT also significantly reduced overexpressions of HIF-1 $\alpha$ , TNF- $\alpha$  and IL-1 $\beta$  and increased the amounts of VEGF, NGF and Schwann cells.

**Conclusions.** LLLT can modulate HIF-1 $\alpha$  activity and may represent a novel, clinically applicable therapeutic approach for improvement of tissue hypoxia/ischemia and inflammation in nerve entrapment neuropathy as well as for promotion of nerve regeneration, which may lead to sufficient morphologic and functional recovery of the peripheral nerve.

**Key Words:** Chronic constrictive injury; Low-level laser therapy; Hypoxia-inducible factor 1 $\alpha$ ; Neuropathic pain; Functional recovery

## INTRODUCTION

Neuropathic pain is a common sequela initiated by a primary lesion of the nervous system.<sup>1-2</sup> The relationship between proinflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin 1 (IL-1) released by inflammatory cells and the development of neuropathic pain has been identified in previous studies.<sup>3-6</sup> These results support that nerve inflammation plays an important role in the development and progression of neuropathic pain. Chronic constriction injury (CCI) of the sciatic nerve with loose ligatures is the most widely used model for neuropathic pain,<sup>7-8</sup> simulating the clinical condition of chronic nerve compression in nerve entrapment neuropathy or spinal root irritation by a lumbar disk herniation.<sup>1</sup>

Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is increased in conditions of hypoxia, ischemia and inflammation.<sup>9</sup> HIF-1 $\alpha$  is essential in maintaining inflammatory processes by promoting the production of proinflammatory cytokines, including TNF- $\alpha$  and IL-1 $\beta$ .<sup>10</sup> HIF-1 $\alpha$  has been identified as a pivotal transcription factor linking the inflammatory pathways.<sup>11</sup> Inhibition and/or down-regulation of these molecules may exert anti-hypoxic and anti-inflammatory effects. Therefore, inhibiting HIF-1 $\alpha$  accumulation may be a novel therapeutic strategy for neuropathic inflammation.

Many studies have also reported positive effects of low-level laser therapy (LLLT) for promoting the repair processes of peripheral nerve by increasing vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) secretions,<sup>12-17</sup> and by inhibiting the inflammation through reduction of pro-inflammatory cytokines.<sup>18</sup> However, to date, there is little evidence directly supporting the anti-allodynia effects of LLLT in neuropathic pain. For this study, we hypothesized that LLLT can decrease pro-inflammatory cytokines, reduce HIF-1 $\alpha$  accumulation, and then promote expressions of VEGF and NGF in the sciatic nerve proximal to the site of CCI on improvement of neuropathic pain and functional recovery.

## MATERIALS AND METHODS

### General Design

Animals (n=40) were divided randomly into four groups (Figure 1): (1) the CL group (n=10) including CCI animals with LLLT; (2) CsL group (n=10) including CCI animals with sham-irradiated LLLT; (3) sCL group (n=10) including sham-operated CCI animals with LLLT; and (4) sCsL group (n=10) including sham-operated CCI animals with sham-irradiated LLLT. Treatments of LLLT or sham-irradiation were given for 7 consecutive days.

Mechanical paw withdrawal threshold (MPWT), sciatic functional index (SFI), tibial functional index (TFI), peroneal functional index (PFI), histology, immunohistochemistry and immunoassays were assessed. Pain and functional assessments were performed the day before (pre-op, at day 0), immediately after operation (post-op, at day 1), at 7 days (7d post-op, at day 7) after surgery and after the 7-day treatment (post-tr, at day 14). Animals were sacrificed for assessments of histopathology and immunoassays the day after completing the treatments. A flow diagram of the experimental design is presented in Figure 1.

## **Animals**

Experiments were performed on adult male Sprague–Dawley rats (SD, 250 to 300 g, purchased from BioLASCO Co., Ltd, Taiwan). The animals were kept on an artificial 12-h light–dark cycle in a University Animal Center. The food and water were available ad libitum. The ethical guidelines of the International Association for Study of Pain in Animals were followed.<sup>19</sup> All experimental procedures were approved by the China Medical University Committee on Animal Care and Use.

## **Chronic Constriction Injury of Sciatic Nerve**

Following the procedure originally proposed by Bennet and Xie,<sup>7</sup> surgery was performed under anesthesia with 4% isoflurane in liquid form for inhalation (AErrane, Baxter Healthcare of Puerto Rico, PR). Using an operating microscope, the sciatic nerve on one randomly selected side was exposed by skin incision along the femur and separation of biceps femoris and gluteal muscles. At the middle third of the sciatic nerve, four ligatures with 4-0 chromic gut thread (Ethicon, USA) were tied loosely around the nerve with inter-ligation spacing of about 1 mm. The wound of muscle layers and skin were then sutured with 4/0 reabsorbable suture (Ethicon, USA) to allow recovery. Sham-operated CCI animals underwent the same procedures with branches dissociated but without any nerve lesion.

## **Low-Level Laser Irradiation**

Seven days after surgery, a continuous 660-nm Ga-Al-As diode laser (Aculas-Am series, Multi-channel LLLT system)<sup>a</sup> was used for treatment. After sterilization, the hand-held delivery probe was placed lightly on the skin surface directly above the loose nerve ligation site at 4 spots / per area. The spot size was approximately 0.2 cm<sup>2</sup>. The output power of the laser irradiation was 30 mW per session for 60 sec/ per spot for 7 consecutive days. The energy density was 9 J/cm<sup>2</sup>. The output of the equipment was routinely checked by the Laser Check Power Meter.<sup>b</sup> A similar procedure was applied to the control group with sham-irradiated LLLT with the power set at 0.

## **Mechanical Allodynia**

The mechanical allodynia was assessed with MPWT by measuring nociceptive thresholds to von Frey filaments stimulation at pre-op, post-op, 7d post-op and post-tr. The test consisted of evoking a hind paw flexion reflex with an electronic von Frey anesthesiometer<sup>c</sup> adapted with a 0.5 mm<sup>2</sup> polypropylene tip. In a quiet room, the rats were placed in acrylic cages (32 × 22 × 27 cm<sup>3</sup>) with a wire grid floor for 30 min habituation prior to testing. The polypropylene tip was perpendicularly applied to the central area of the hind paw with sufficient force to bend the filaments into an “S” shape for 3-4 sec. The test consisted of poking a hind paw to provoke a flexion reflex followed by a clear flinch response after paw withdrawal. Testing was initiated with the filament corresponding to 20 log of force (g). The filaments were applied with a gradual increase in pressure until a withdrawal reflex response observed. The weakest filament able to elicit a response was taken to be the MPWT (g). The-final value for the response was obtained by averaging five measurements.

## **Assessments of Functional Recovery**

The degree of recovery was monitored by evaluating the rats' walking patterns measured with SFI, TFI, and PFI according to the method described by Bain et al which had been well established.<sup>20</sup> Before the recording, a few conditioning trials were performed to accustom the animals to the track. All animals underwent preoperative walking-track analysis. The plantar surfaces of both hind paws were wetted with red ink to obtain clear footprints when they walked along a specially designed alley (84 cm length × 8.5 cm width) lined with scaled paper. Recordings continued until five measurable footprints had been collected. The data used for calculations were taken from the footprint as follows: (1) distance from the heel to the third toe, the print length (PL); (2) distance from the first to fifth toe, the toe spread (TS); and, (3) distance from the second to the fourth toe, the intermediary toe spread (IT). All three measurements were taken from the experimental (<sup>E</sup>) and normal (<sup>N</sup>) sides. Prints were then calculated using the following formulae: (1)  $SFI = -38.3 \left( \frac{[{}^E PL - {}^N PL]}{{}^N PL} \right) + 109.5 \left( \frac{[{}^E TS - {}^N TS]}{{}^N TS} \right) + 13.3 \left( \frac{[{}^E IT - {}^N IT]}{{}^N IT} \right) - 8.8$ ; (2)  $TFI = -37.2 \left( \frac{[{}^E PL - {}^N PL]}{{}^N PL} \right) + 104.4 \left( \frac{[{}^E TS - {}^N TS]}{{}^N TS} \right) + 45.6 \left( \frac{[{}^E IT - {}^N IT]}{{}^N IT} \right) - 8.8$ ; (3)  $PFI = 174.9 \left( \frac{[{}^E PL - {}^N PL]}{{}^N PL} \right) + 80.3 \left( \frac{[{}^E TS - {}^N TS]}{{}^N TS} \right) - 13.4$ . Values of these tests equal to -100 indicated total impairment of the sciatic, posterior tibial and peroneal nerves, whereas SFI, TFI and PFI oscillating around 0 were considered to reflect normal function.<sup>20</sup>

## **Sciatic Nerve Obtainment and Tissue Preparations**

After completing the treatments at day 14, rats were sacrificed after being deeply

anaesthetized with saturated KCl (300 g/ml, i.p.), then sciatic nerve segment was harvested, which included the four ligatures site and 1 cm proximal to this site. The biopsied nerve specimens were divided into two portions for histopathology and immunoassays. For histopathological assessments, nerve specimens were fixed in 10% neutral formalin, and embedded in paraffin for 12 h at room temperature. For immunoassays, specimens were homogenized in T-PER™ (Tissue Protein Extraction Reagent, Pierce Chemical Co., IL, USA) and the complete cocktail of protease inhibitors (Sigma, NY, USA). After centrifuging, the supernatant was extracted and stored at  $-80^{\circ}\text{C}$ .

### **Histopathological, Immunohistochemical and Immunofluorescent Stainings**

The specimens were submitted to diafanization with xylene, then dehydrated by graded ethanol, embedded in paraffin and cut in 4- $\mu\text{m}$ -thick sections longitudinally using a microtome. Histopathologic changes were evaluated on sections stained with hematoxylin and eosin (H&E, Muto Pure Chemicals Co., Ltd., Tokyo, Japan) to determine infiltration of inflamed cells in nerves. Slides were examined by a light microscope and photographed. The area of inflamed cell and nerve nuclei was measured in a  $200\times$  magnification field by an ImageScope program.<sup>d</sup>

For immunohistochemical staining, the slides were first incubated overnight at  $4^{\circ}\text{C}$  with the monoclonal mouse antibodies, including anti-HIF-1 $\alpha$  (1:200, Thermo, CA, USA), anti-monocytes/macrophages (ED1, 1:200, Millipore, CA, USA) primary antibodies, with the polyclonal rabbit antibodies, including anti-Schwann cells (S100, 1:400, DakoCytomation, Denmark) and anti-VEGF (1:200, Abbiotec, CA, USA) primary antibodies, as well as with rabbit monoclonal anti-NGF- $\beta$  (1:2500, Millipore, CA, USA) primary antibody. The nerve sections were then incubated with biotinylated goat anti-mouse and goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 hour at room temperature. After washing, sections were incubated with a streptavidin-horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Finally, sections were visualized as brown precipitates yields using 3,3'-diaminobenzidine (DAB, 0.2 mg/ml, Pierce, Rockford, IL, USA) as a substrate and then counterstained with hematoxylin. Slides were examined at a minimum of five sections in the more representative fields using a light microscope and then photographed. The area sizes of positive nuclear and cytoplasmic staining cells for HIF-1 $\alpha$ , ED1, S100, VEGF and NGF were measured in a  $200\times$  magnification field using the ImageScope program.<sup>d</sup> Results are expressed as the proportion (%) of positive immunoreactive area per total stained area.

To observe coexpression of HIF-1 $\alpha$  with infiltrated inflammatory cells in the injured nerve, we incubated the sections with rabbit polyclonal anti-HIF-1 $\alpha$  (1:200, Santa Cruz

Biotechnology, CA, USA) and mouse monoclonal anti-monocytes/macrophages (ED1) (1:200, Millipore, CA, USA) overnight at 4°C. Sections were then incubated with the respective secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), goat anti-rabbit IgG fluorescein-conjugated (FITC, 1:1000) and goat anti-mouse IgG rhodamine-conjugated (TRITC, 1:1000) secondary antibodies for 2 hours at room temperature. After washing, sections were incubated with a streptavidin-horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and then counterstained with 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes, Invitrogen Corporation, Carlsbad, CA, USA) to reveal cell nuclei. Images were obtained using a conventional fluorescence microscope (Fluoview X).<sup>e</sup> All of quantitative image analyses were assessed by two independent observers who were blinded to the origin of the sections to avoid bias from interobserver variability.

### **Enzyme-Linked Immunosorbent Assay**

The amounts of TNF- $\alpha$  and IL-1 $\beta$  concentrations in the supernatants were determined using the DuoSet<sup>®</sup> ELISA Development kit (R&D Systems, Minneapolis, MN, USA). Nerve extracts were incubated in 96-well plates coated with mouse anti-rat TNF- $\alpha$  and goat anti-rat IL-1 $\beta$ . Biotinylated anti-rat TNF- $\alpha$ , anti-rat IL-1 $\beta$  and then streptavidin-HRP were added and incubated in accordance with the manufacturer's instructions. After washing, a NeA-Blue (Tetramethylbenzidine) Substrate solution (Clinical Science Products, Inc., Mansfield, MA, USA) was added. The enzyme reaction was terminated by adding stop solution (2N H<sub>2</sub>SO<sub>4</sub>). The levels of TNF- $\alpha$  and IL-1 $\beta$  were assessed by a reader (Multiskan EX Microplate Photometer)<sup>f</sup> using a 450 nm filter and normalized with an abundance of standard solution. Data were then analyzed using Thermo Scientific Ascent Software<sup>f</sup> and a four-parameter logistics curve-fit.

### **Western Blot Analysis**

Protein determination was performed by modified Lowry protein assays. Equal amounts of protein were loaded and separated in 10% Tris-Tricine SDS-PAGE gels. The resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membranes<sup>g</sup>. The membranes were blocked in 5% non-fat milk for 1 hour at room temperature, and incubated overnight at 4°C with mouse monoclonal anti-HIF-1 $\alpha$  (1:500, Novus Biologicals, CA, USA), rabbit polyclonal anti-VEGF antibody (1:2500, Abbiotec, CA, USA), and rabbit monoclonal anti-NGF- $\beta$  (1:2500, Millipore, CA, USA) primary antibody. The blots were then incubated with the horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG secondary antibody (1:20000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 hour at room temperature. Signals were finally visualized using enhanced chemiluminescence detection system (Fujifilm LAS-3000 Imager),<sup>h</sup> and the blots were exposed to X-ray films. All Western blot analyses were performed at least three times, and consistent results were

obtained. Immunoreactive bands were analyzed using a computer-based densitometry Gel-Pro Analyzer (version 6.0).<sup>i</sup> Grey levels, obtained by densitometric analysis of immunoreactive bands, were normalized on  $\beta$ -actin.

### **Statistical Analysis**

Results were averaged for each group and values were expressed as mean  $\pm$  S.E.M. The data obtained from MPWT, SFI, TFI and PFI were analyzed using mixed-design, two-way repeated-measures ANOVA performed with group as a between-subjects factor and time as a within-subjects factor. The Bonferroni adjustment was examined post hoc for multiple comparisons at individual time points between groups. One-way ANOVA was performed for comparison of individual group means for assessing parametric results of histopathology and immunoassay. The Dunnett test was performed for multiple comparisons between experimental and control groups at the post-tr time point. A *P* value of  $< .05$  was considered statistically significant. All data were analyzed using SPSS version 10.0 for Windows.<sup>j</sup>

## **RESULTS**

### **Effects of Low-Level Laser Therapy on Mechanical Allodynia**

MPWT was significantly decreased at post-op and 7d post-op conditions in animals with CCI comparing to the pre-op condition (both were  $P < 0.001$ ). In animals with sham-operated CCI, MPWT of post-op was also significantly lower ( $P < 0.0001$ ) than that of pre-op condition, whereas there was no significant difference between the 7d post-op and pre-op condition ( $P=0.36$ ). There were also significant differences among the four groups at each time point (all were  $P < 0.0001$ , Figure 2A).

Comparing to 7d post-op values, significant improvement in MPWT after treatment was noticed in CL group ( $P < .0001$ ), but not in all sham groups of CsL, sCL and sCsL (all were  $P > .05$ ). After treatment, significantly higher MPWT existed in CL group than those in CsL groups ( $P < .0001$ ). However, no significant difference was observed between sCL and sCsL groups ( $P=0.98$ ).

### **Effects of Low-Level Laser Therapy on Functional Recovery**

SFI, TFI and PFI values were around 0 before surgery, and decreased significantly after surgery in all groups ( $P < .001$ ). In animals with CCI, SFI and TFI continued to decrease at 7d post-op compared with those of post-op (SFI:  $P=0.83$ ; TFI:  $P=0.99$ ), but PFI recovered significantly ( $P < .0001$ ). However, at 7d post-op, animals with sham-operated CCI had PFI



values increased and approached the pre-surgery values ( $P = 0.99$ ), with SFI and TFI values also increased significantly compared with post-op values (both were  $P < .0001$ , Figure 2B-D).

Comparing to 7d post-op values, SFI, TFI and PFI values were significantly improved after treatment in CL group (SFI:  $P=0.001$ ; TFI:  $P=0.003$ ; PFI= $0.03$ ), but not in all sham groups of CsL, sCL and sCsL (all were  $P > .05$ ). After treatment, significantly higher values of SFI, TFI and PFI existed in CL group than those CsL groups (SFI:  $P=0.001$ ; TFI:  $P=0.004$ ; PFI:  $P=0.002$ ).

### **Effects of Low-Level Laser Therapy on Inflammation and Cytokines**

In H&E study, there was pronounced infiltration of immune cells at the site of CCI injury as compared with the sham-operated site (Figure 3A, 3B, 3C, 3D). The percentage of nuclei was significantly decreased, showing less inflammation and cell infiltration in CL groups when compared with CsL group (Figure 3G). Similar results were found for ED1 immunoreactivity which showed significant increases in CsL group, but was reduced in CL group (Figure 3E, 3F and 3H).

There were significantly higher levels of TNF- $\alpha$  and IL-1 $\beta$  in CsL groups in comparison with those in sCsL and sCL groups (both were  $P < .0001$ ). There was a significant reduction of these cytokines in the CL group when compared with CsL groups ( $P < .0001$ ), but no significant difference was found when compared with those of sCL (TNF- $\alpha$ :  $P=0.29$ ; IL-1 $\beta$ :  $P=0.39$ ) or sCsL (TNF- $\alpha$ :  $P=0.33$ ; IL-1 $\beta$ :  $P=0.28$ ) groups (Figure 4).

### **Effects of Low-Level Laser Therapy on HIF-1 $\alpha$**

There were sparse HIF-1 $\alpha$ -positive cells in sCL and sCsL groups (Figure 5A, 5B) and no significant differences were found among these groups (both were  $P > .05$ , Figure 5G). In the CsL group, overexpression of HIF-1 $\alpha$  immunoreactivity was observed and localized in both the nucleus and cytoplasm of the injured samples at higher-power magnification (Figure 5C). The accumulation of HIF-1 $\alpha$ -positive cells was decreased significantly in CL group when compared with CsL group ( $P=0.006$ , Figure 5D). Double staining with HIF-1 $\alpha$  and ED1 could show the ED1 immunoreactive cells which were morphologically consistent with macrophages, mainly by inflammatory infiltration of the inflamed nerve, and coexpressed by the specific HIF-1 $\alpha$  immunoreactivity. The number of double positive cells was decreased in CL groups when compared with those in CsL group (Figure 5E and 5J). The observed HIF-1 $\alpha$  expressions were further supported at the protein level assay by Western blotting, showing levels of HIF-1 $\alpha$  as gray density percentages (normalized on  $\beta$ -actin) in the form of a

representative Western blotting (Figure 6H). Significantly higher levels of HIF-1 $\alpha$  level were found in CsL groups than in CL, sCsL and sCL groups (all were  $P < .0001$ ). The protein levels of HIF-1 $\alpha$  was significantly decreased in CL group in comparison with CsL groups ( $P=0.006$ ) and approximated the levels of sCL control group ( $P=0.064$ ). No significant differences were observed between sCL and sCsL groups ( $P > .05$ ).

### **Effects of Low-Level Laser Therapy on VEGF, NGF and Schwann Cells**

As expected, there were significant increases of VEGF and NGF in CL groups compared with those in CsL group (VEGF:  $P=0.009$ ; NGF:  $P=0.002$ , Figure 6B, 6C, 6E and 6F). There were no significant differences of VEGF and NGF expression between sCL and sCsL groups (both were  $P > .05$ ). After CCI, the expressions of these factors in the injured sciatic nerve were slightly increased in CsL group as shown in Figures 6A and 6D, but the difference was of non-significant when compared with those of sCsL groups (NGF:  $P=0.9$ ; VEGF:  $P=0.22$ ).

Furthermore, as demonstrated in Figure 6I and 6J, the observed VEGF and NGF immunoreactive expressions could be further supported at the protein level by Western blotting. There were significant increases of levels of VEGF and NGF in CL group when compared with those of CsL groups (VEGF:  $P=0.009$ ; NGF:  $P=0.002$ ). The protein levels of VEGF and NGF in CsL group also showed a slight elevation over 14 days after CCI surgery but the calculation was not significant when compared with those of sCsL groups (NGF:  $P=0.18$ ; VEGF:  $P=0.07$ ). No significant differences were observed between sCL and sCsL groups (both were  $P=1.0$ ).

Using S100 immunohistochemistry for Schwann cells, the S100 expression was decreased in injured nerve in CsL group (Figure 6G), but increased in CL group (Figure 6H). The S100 expression in CsL group was significantly lower than the values in CL ( $P=0.005$ ), sCL ( $P=0.035$ ) and sCsL ( $P=0.027$ ) groups (Figure 6I).

## **DISCUSSION**

The most important finding in this study is the evidence of laser effects on the recovery of pain and walking function accompanied with the improvement of inflammation in rats after chronic compression of sciatic nerve. We used an animal model of CCI and had comparable findings similar to previous reports.<sup>8, 21-22</sup> In this animal model, we demonstrated pain and functional impairment in animals with CCI. We also found overexpressions of HIF-1 $\alpha$ , TNF- $\alpha$  and IL-1 $\beta$  after CCI without LLLT.

In the current study, we demonstrated that 660nm-GaAlAs-LLLT at a dose of 9 J/cm<sup>2</sup> significantly reduced neuropathic allodynia in CCI rats. The result was similar to those of a previous report that demonstrated laser could effectively reduce pain in rats with sciatica.<sup>23</sup> Clinical studies of carpal tunnel syndrome also demonstrated significant improvement in pain and nerve conduction in patients undergoing LLLT over the carpal tunnel area.<sup>24-25</sup> Our results also showed that the LLLT significantly promoted functional recovery as evidenced by increases in the SFI, TFI and PFI. These results are consistent with the findings of a previous study that demonstrated LLLT was effective in promoting early functional recovery of sciatic crush injury.<sup>26</sup>

Our results demonstrated that the infiltration of inflamed cells and the release of TNF- $\alpha$  and IL-1 $\beta$  were significantly reduced after LLLT. This result is similar to findings of previous studies with a rat model of carrageenan-induced inflammation.<sup>27-28</sup> Therefore, the alleviation of neuropathic pain treated with LLLT in this study was probably due to the reduction of inflammation and pro-inflammatory cytokines of injured nerve tissue. Accumulation of HIF-1 $\alpha$  protein has been found to exist following inflammation, probably induced by TNF- $\alpha$  and IL-1.<sup>11, 29-30</sup> In this study, it was found that the accumulation of HIF-1 $\alpha$  in damaged nerve tissues was suppressed after LLLT. LLLT also reduced HIF-1 $\alpha$  expression in macrophages which coordinate chronic inflammation and immune responses. Our results are similar to a recent study of a mouse infection model for wound healing which demonstrated high immunoreactivity for HIF-1 $\alpha$  in untreated lesions, but little immunoreactivity in laser-treated lesions<sup>31</sup>. We postulate that this finding may explain the ability of laser radiation for eliminating HIF-1 $\alpha$  accumulation and then stabilizing its activity, thereby stimulating aerobic cell metabolism, accelerating tissue repair and promoting functional recovery.

Angiogenesis is an essential component of nerve re-growth prior to nerve regeneration.<sup>32-33</sup> VEGF, a potent growth factor for angiogenesis, also plays an important role in proliferation of Schwann cells, nerve repair and motor performance.<sup>34-35</sup> Moreover, stabilization of HIF-1 $\alpha$  in a mouse with diabetes enhances wound healing and increases VEGF production.<sup>36</sup> Our results demonstrated that LLLT could facilitate a prominent increase of VEGF immunoreactivity compared with that obtained by sham-irradiation. This effect was probably achieved through the stabilization of HIF-1 $\alpha$  protein activity. A study with similar findings to our current study showed that LLLI could stimulate proliferation and differentiation of bone marrow-derived mesenchymal stem cells associated with increased VEGF secretion,<sup>17</sup> indicating that LLLT can accelerate the tissue healing process by stimulating VEGF.

NGF may act on the regeneration and growth of axonal processes to promote the survival of sensory neurons and reverse degeneration of myelin.<sup>37-39</sup> In accordance with these previous findings,<sup>12-17</sup> our results showed that the elevation of NGF protein by LLLT was greater than that by sham-irradiation. Moreover, in this study, an increase of S100 immunoreactivity was also found after LLLT, indicating an increase in Schwann cells and these changes may be attributed to improvement of functional recovery. Therefore, improvement of neural function could also be achieved by application of LLLT which can increase protein levels of VEGF and NGF to repair the myelin sheath in the injured nerve tissues.

## Study Limitations

Our study found that 660nm-GaAlAs-LLLT at a dose of 9 J/cm<sup>2</sup> significantly improved neuropathic pain and functional recovery in CCI rats. The molecular mechanisms of this effects involved reduction of TNF- $\alpha$ , IL-1 $\beta$  and HIF-1 $\alpha$  accumulation and activation of VEGF and NGF expression. However, it is still unknown whether this effect exists for other wavelengths or modes of laser with the same energy intensity, and therefore, further studies are necessary in the future. In addition, there are several factors relevant to inflammation and oxidative stress, including nitric oxide (NO), cyclooxygenase enzymes-2 (COX-2) and tumor protein 53 (p53) which have been shown to either be activated in peripheral neuropathy or result in up-regulation of HIF-1 $\alpha$ .<sup>40-41</sup> But the mechanism of the effect of LLLT on several HIF-1 $\alpha$ -induced genes encode proteins in the present study is unknown. Because stabilization of HIF-1 $\alpha$  protein expression as a regulator of gene expression in tissues is required for the establishment of normal physiological systems,<sup>42</sup> we hypothesize that the mechanism reducing pain and stimulating nerve repair could be induced by influencing HIF-1 $\alpha$  and other HIF-1 $\alpha$ -target genes. Further research is mandatory to explore this pathway. Lack of electrophysiological assessments for the effect of LLLT is another deficiency of this study. Further study accompanies with nerve conduction measurements on CCI nerve after LLLT is necessary.

## CONCLUSIONS

Results in this study support our postulation that LLLT applied transcutaneously to the CCI nerve can suppress inflammation-induced TNF- $\alpha$ , IL-1 $\beta$  and HIF-1 $\alpha$  accumulation to control the neuropathic pain and elevate the levels of VEGF and NGF in injured nerve thereby promoting functional recovery and nerve regeneration. It has been suggested that LLLT can modulate HIF-1 $\alpha$  activity and may represent a novel therapeutic approach for improvement of tissue hypoxia/ischemia in nerve entrapment neuropathy as well as for acceleration of regeneration process, which may lead to sufficient morphologic and functional recovery of the peripheral nerve.

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