中 國 醫 藥 大 學
物理治療學系復健科學碩士班
碩士論文

利用兩種運動訓練減緩大白鼠坐骨神經慢性壓迫性損傷所引起的周邊神經病理性疼痛及神經損傷

Two exercise training reduce peripheral neuropathic pain and protect nerve damage following chronic constriction injury of sciatic nerve in rats

指 導 教 授：陳 郁 文 博士 (Yu-Wen Chen)

研 究 生：李 泳 璁 (Yung-Tsung Li)

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中文摘要

研究背景:

周邊神經損傷引起的神經病理性疼痛使用藥物治療具有不錯的治療效果，但是會產生副作用。運動影響神經病理性疼痛的機轉還不是很清楚。所以本篇研究為了探討運動訓練，一種非藥物治療的方式，是否能提供神經病理性疼痛較佳的治療效果。

材料和方法:

本實驗使用雄性大白鼠接受慢性壓迫性神經損傷建立神經病理性疼痛模式，並且接受運動治療。將所有的雄性大白鼠分成七個組別: 控制組、假手術組、假手術游泳或跑步機運動治療組、神經損傷組、神經損傷游泳或跑步機運動治療組。首先，觀察運動治療後其體重、熱覺過敏和觸覺過敏的變化。並且使用蛋白質轉漬法或酵素連結免疫吸附分析法偵測運動治療後熱休克蛋白 72 和原發炎細胞介素在坐骨神經的表現量。同時，利用組織病理性檢查法觀察坐骨神經受損接受運動治療後型態學的變化。

結果:

假手術游泳或跑步機運動治療組、神經損傷組、神經損傷游泳或跑步機運動治療組的體重比控制組和假手術組高。一直到手術後第 39 天，控制組、假手術組、假手術游泳或跑步機運動治療組都沒有發生熱覺過敏和觸覺過敏的現象。神經損傷接收游泳和跑步機運動治療 21 天後明顯緩解熱覺過敏和觸覺過敏的現象。神經損傷接收游泳和跑步機運動治療 22 和 40 天後降低 tumor necrosis factor-α 和 interlukin-1β 在坐骨神經的表現量。神經損傷後接受跑步機運動治療 22 和 40 天，熱休克蛋白 72 的表現量增加。

結論:

由以上的研究結果發現游泳和跑步機運動降低熱覺過敏、觸覺過敏、tumor necrosis factor-α 和 interlukin-1β 的表現。神經損傷後給予跑步機運動治療增加坐骨神經熱休克蛋白 72 的表現量，但在神經損傷後給予游泳運動治療熱休克蛋白 72 並無明顯增加。此實驗顯示運動治療能夠提供神經病理性疼痛不錯的治療效果。游泳和跑步機運動治療能夠促進坐骨神經慢性壓迫性損傷後型態學和感覺系統的恢復。

關鍵字：游泳、跑步機、慢性壓迫性損傷、神經病理性疼痛、Tumor necrosis factor-α、Interlukin-1β、 熱休克蛋白 72、型態學

英文摘要(ABSTRACT)

Background: Pharmacotherapies provide an effective efficacy for treatment of neuropathic pain, but they produce side effects. The mechanism of exercise on neuropathic pain is not clear. The aim of this study was to investigate whether exercise training, a non-pharmacotherapy method, provide beneficial effects on neuropathic pain.

Materials and Methods: Male Sprague Dawley rats that suffered for chronic constriction injury (CCI) with/without exercise were used. All rats were divided into 7 groups: control, sham operated, sham operated with swimming or treadmill exercise, CCI, CCI with swimming or treadmill exercise. We observed body weight, thermal hyperalgesia and mechanical allodynia in all groups. The expression of heat shock protein 72 (HSP72) and pro-inflammatory cytokines (tumor necrosis factor-α and interlukin-1β) in sciatic nerve were detected by immunoblotting or ELISA, respectively. We also investigated the morphological change of sciatic nerve after CCI treatment.

Results: The body weight in sham operated with swimming or treadmill exercise, CCI, CCI with swimming or treadmill exercise groups was heavier than that in control and sham operated groups. In control, sham operated, sham operated with swimming or treadmill exercise groups, rats showed no thermal hyperalgesia and mechanical allodynia until day 39 after CCI-treatment. CCI rat with swimming (hyperalgesia, $P<0.05$; allodynia, $P<0.01$) or treadmill exercise (hyperalgesia, $P<0.05$; allodynia, $P<0.01$) attenuated thermal hyperalgesia and mechanical allodynia significantly, when compared with CCI rats with exercise 21 days after CCI-treatment. We also found that swimming and treadmill exercise reduced tumor necrosis factor-α (TNF-α) and interlukin-1β (IL-1β) expression

in sciatic nerve 22 or 40 days after CCI-treatment. HSP72 expression in sciatic nerve increased in CCI with treadmill exercise group ($P< 0.05$), but not in CCI with swimming exercise group, compared with CCI group 22 or 40 days after CCI-treatment. The morphological improvements seen in sciatic nerve in CCI with treadmill or swimming exercise group, compared with CCI group on 22 or 40 days after CCI-treatment.

Conclusions: This study indicated that swimming and treadmill exercise partially ameliorates thermal hyperalgesia, mechanical allodynia, TNF-α and IL-1β expression in sciatic nerve. Treadmill exercise, but not swimming exercise, increased HSP72 expression in sciatic nerve of CCI with exercise rats, compared with CCI without exercise rats. Both of treatment with swimming and treadmill exercise promotes restoration of morphological and functional properties following CCI.

Key Words: Swimming, Treadmill, Chronic constriction injury, Neuropathic pain, Tumor necrosis factor-α, Interlukin-1β, Heat shock protein 72, Morphology

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List of abbreviations and symbols

SO	sham operation
SOTE	sham operated with treadmill exercise training
SOSE	sham operated with swimming exercise training
CCI	chronic constriction Injury
CCITE	chronic constriction injury with treadmill exercise training
CCISE	chronic constriction injury with swimming exercise training
HSP	heat shock protein
TNF-α	tumor necrosis factor - alpha
IL-1β	interlukin-1 beta
D22	Day 22 after CCI-treatment
D40	Day 40 after CCI-treatment

1. Introduction

Neuropathic pain disturbing ocean of people worldwide has become one of the most notable health problems. They attenuate the patient's ability to work, walk, sleep, even the quality of life (Kuphal et al. 2007). Clinically, patients with neuropathic pain following nerve injury often complain of ongoing burning pain as well as pain to light touch (Meyer and Ringkamp 2008). Although many pharmacotherapies (such as antidepressants, antiepileptics) are effectively available for neuropathic pain, these drugs produce side effects (Saarto and Wiffen 2005). We thought that non- pharmaco-therapies are better to treat neuropathic pain.

Recently, numerous studies indicate that exercise decreases symptoms of acute pain in human (Kemppainen et al. 1998; O'Connor and Cook 1999; Hoffman et al. 2005). Exercise has beneficial effects on chronic disease, presents the anti-inflammatory effect, and reduces neuropathic pain (Woods et al. 2006; Kuphal et al. 2007). However, only a small number of studies report exercise-induced analgesia in the setting of inflammatory pain, and even fewer relate long-term exercise to reductions in neuropathic pain. Indeed, there is an increase in heat shock protein 70 (HSP70) after treadmill running in the rodent muscles (Noble et al. 2008). Furthermore, the exercise pretraining can induced the HSP72 expression, against the cerebral ischaemia and damage to cerebral neurons in rodent model (Chen et al. 2007). In addition, HSP70 protect neurons from the damaging effects of stressors by interrupting inflammation responses (Noble et al. 2008).

Neuropathic pain provokes varying degrees of local inflammatory responses and overexpression of inflammatory cytokines in locally activated of resident immune cells and recruited and proliferated

of marcophages, mast cells, neutrophils, Schwann and glia cells (Martucci et al. 2008). Pro-inflammatory cytokines such as TNF-α, which is released into the local environment following nerve injury, and play an important role in the initiation and maintenance of neuropathic pain (Marchand et al. 2005). Inhibition of TNF reduces hyperalgesia in animal models of neuropathic pain, like CCI and partial nerve transaction (Sommer et al. 2001). Neutralizing antibodies to interleukin 1-receptor attenuate pain associated behavior in mice with experimental neuropathy (Sommer et al. 1999). There are many studies applied pro-inflammatory cytokines (e.g., tumor necrosis factor-α and interlukin-1β) induces pain behavior (Fukuoka et al. 1994; Junger and Sorkin 2000; Schafers et al. 2003; Zelenka et al. 2005), and that treatment with anti-inflammatory cytokines or inhibitors of pro-inflammatory cytokines relieves pain (Sommer 1999; Milligan et al. 2005; Hao et al. 2006; Schafers and Sommer 2007) supporting the concept that pro-inflammatory induce and maintain neuropathic pain.

The aim of this study was to investigate whether exercise training, a non-pharmacotherapy method, reduced peripheral neuropathic pain and protect nerve damage following chronic constriction injury of sciatic nerve in rats. Thermal hyperalgesia, mechanical allodynia, TNF-α, IL-1β and HSP72 were detected in CCI rats with/without treadmill or swimming exercise.

2. Materials and methods

2.1. Animals

Experiments were performed on 250–300 g male Sprague–Dawley rats (National Laboratory Animal Center, Taipei, Taiwan). Rats were housed in a climate controlled room maintained at 21 ℃ with approximately 50% relative humidity in the Animal Center of China Medical University. Lighting was on a 12-h light–dark cycle (light on at 8:00 a.m.), with food and water available ad libitum up to the time of testing. Effort was made to minimize discomfort and reduce the number of animals used. All studies were conducted following the ethical guidelines of IASP ethical guidelines (Zimmermann 1983).

2.2. Drugs, reagents and instruments

2.2.1 Drugs and reagents

Item	**Corporation**
3/0, 4/0 black silk	Unik surgical sutures MFG. CO., Taiwan
96 well microtiter plate	Costar
1% Eosin Y solution, Hematoxylin	Muto Pure Chemicals Co, LTD
10% formaldehyde solution in aqueous phosphate buffer	Mallinckrode chemicals
Ammonia	Panreac
Chemiluminescence ECL western Blotting luminal Reagent, Horseradish peroxidase-conjugated goat anti-mouse secondary antibody	Santa Cruz, CA, USA
Heparin	B. Braun Melsungen AG, Germany

Mouse monoclonal anti-HSP72 antibody, SPA 810	StressGen Biotechnologies, Canada
$Na_2HPO_4 \cdot 12H_2O$	Fluka
NaCl	Sigma, St. Louis, MO
PVDF 0.45μm pore size	Millipore, Bedford, MA
Slides	Thremo Scientific
Skim milk	Difco, Detroit, MI
Tris Base	MDBio, Inc
Tissue Protein Extraction Reagent (T-PER)	Pierce Chemical Co., USA
TNF-α DuoSet® ELISA Development kit, IL-1β DuoSet® ELISA Development kit, DY	R&D Systems, Minneapolis, MN
Urothane, Protease inhibitor, 2N H_2SO_4, KCl, KH_2PO_4, Tween 20	Sigma, St. Louis, MO
Xylene	J.T. Baker
Zoteil 50	Virbac Laboratories, France

2.2.2 Instruments

Item	**Corporation**	**Others**
Chemiluminescence detection system	Fujifilm, Tokyo, Japan	LAS-3000
Clear plexiglass chamber	Made in Taiwan	22 cm wide *22 cm long *13.3 cm tall
Homogenization probe	Tissue Tearor, Polytron, Biospec Products, Inc.	
iEMS Reader	Ascent Software	
Microscope	Zeiss axiophot microscopes	
Plate reader	Molecular Device Spec 383	
Plantar test apparatus	Analgesia Meter, IITC life Science Instruments, Woodland	

	Hills, CA	
Rotary microtome	Histo-Line Laboratories	QP-3268
Running system	Bio-rad	
Swimming pool	Made in Taiwan	29-inch-long*21-inch-wide*23.5-inch-high, HDPE
Treadmill	Chanson, CS-5515, Taiwan	
Tissues embedding	Tissue-Tek® TEC™ 5 Tissue Embedding Console System, Japan	
Transfer apparatus	Bio-rad	
von Frey filament	Analgesia Meter, IITC life Science Instruments, Woodland Hills, CA	
Wire mesh floor	Made in Taiwan	40cm wide *50cm long

2.3. Experimental groups

Eighty-four rats were divided into two major groups: forty-nine rats accepted chronic constriction injury and thirty-five rats accepted sham or no operation. Subsequently, they were grouped into seven groups: (1) eight normal rats (control), (2) sixteen with chronic constriction injury (CCI), (3) sixteen with CCI combine swimming exercise treatment (CCISE), (4) seventeen with CCI combine treadmill exercise treatment (CCITE), (5) ten with sham operated (SO), (6) eight with sham operated combine swimming exercise treatment (SOSE), (7) nine with sham operated combine treadmill exercise treatment (SOTE).

2.4. Chronic constriction injury of sciatic nerve in rats

The animals were deeply anesthetized with Zoteil 50 (50 mg/g body weight, i.p.) and under clean conditions the skin on the lateral surface of the thigh was incised and a section made directly through freed of tissue adhesions about 7 mm of nerve just distal to the greater trochanter. The sciatic nerve was exposed through the biceps femoris muscle exposing the sciatic nerve and its three terminal branches: the sural, common peroneal and tibia nerves. Four ligatures (4/0 black silk) were tied loosely around the sciatic as described by (Bennett and Xie 1988)). The left sciatic nerve was exposed proximal to the sciatic trifurcation and an approximately 7 mm section of the nerve was liberated from the adhesive tissue. Loose ligations were tied around the nerve with four silk sutures and about 1mm inter-ligation spacing. The length of nerve thus affected was 5-6 mm long. Great care was taken to tie the ligatures such that the diameter of the nerve was seen to be just barely constricted. The desired degree of constriction retarded, but did not arrest, circulation through the superficial epineurial vasculature and sometimes produced a small, brief twitch in the muscle surrounding the exposure. The incision of the muscle, the adjacent fascia and the skin were closed with 3/0 silk allowing animals recovery. The incisions were closed with wound clips and the animals returned to their cages for recovery. Sham controls involved exposure of the sciatic nerve with the same procedures and its branches without any lesion.

2.5. Swimming exercise

The swimming exercise training protocol was performed and modified from previously described methods (Kuphal et al. 2007), as illustrated in Table 1. Rats were placed in a plastic containers (29-inch long* 21-inch wide* 23.5-inch high) filled with approximately 17 inches of water

maintained at 37±0.5℃. In the rare instance of such behavior, animals were mildly stimulated to swim by nudging the nape with a pen. This ensured a full session of exercise conditioning. After each exercise session, animals were gently dried with a cloth towel.

2.6. Treadmill exercise

The treadmill exercise training protocol was performed according to previously described methods (Chen et al. 2007; Hung et al. 2008), as illustrated in Table 2. In brief, rats were trained to run on a treadmill (Chanson, CS-5515, Taiwan) 5 days a week for 6 weeks period. Initially, the training program acclimatised rats to run for 15 min 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 min at 1.2 km/hr, 30 min at 1.8 km/hr and 60 min at 1.8 km/hr after 1, 2 and 3 weeks of training, respectively. The work rate of rats on this training protocol is about 70–75% of their maximal oxygen consumption. If rats get hurt on feet of this totally training protocol were withdrawn from the study.

2.7. Evaluation of hyperalgesia and allodynia

The rats were tested for hyperalgesia and allodynia after a period of at least 3 days of habituation to the testing environment and observer. Unless otherwise specificed, behavior tests were conducted for all rats at 2 separate days preceded the surgery for two baseline measurements, then tested after the surgery on day 1, 3, 7, 14, 21, 28, 35 and 39. All measurement s were recorded between 10:00 a.m. and 14:00 a.m.

Thermal hyperalgesia was tested according to the Hargreaves procedure(Hargreaves et al. 1988).

The lateral plantar surface was exposed to a beam of radiant heat through a transparent perspex surface, a Plantar test apparatus (Analgesia Meter, IITC life Science Instruments, Woodland Hills, CA). Briefly, rats were placed individually in clear plexiglass chamber (22 cm long *22 cm wide *13.3 cm tall) and support by a temperature maintained (30 ±1℃) glass sheet allowing to acclimatize. A constant intensity radiant heat source (focused beam of light, beam diameter 0.5 cm and intensity 20 I.R.) was aimed at the midplantar area of the left hindpaw near the tibia nerve and sural nerve innervated. The paw withdrawal latency was recorded. The withdrawal responses evoked by thermal stimulation was determined, including foot lifted, shaking, licking and squeaking. Paw movements associated with weight shifting or locomotion were not counted. The heat stimulation was repeated 3 times at an interval of 5 min for each test and the mean calculated. A maximal automatic cut-off latency of 20 s was used to prevent tissue damage to the hindpaw.

For mechanical allodynia, rats were placed individually in clear plexiglass chamber (22 cm long *22 cm wide *13.3 cm tall), support by a wire mesh floor (40 cm wide *50 cm long) allowing to acclimatize. The electronic von Frey filament (Analgesia Meter, IITC life Science Instruments, Woodland Hills, CA) was applied at the midplantar area of the left hindpaw near the tibia nerve and sural nerve innervated. The paw withdrawal threshold was recorded. The withdrawal responses evoked by mechanical stimulation was determined, including foot lifted, shaking, licking and squeaking. Paw movements associated with weight shifting or locomotion were not counted (Hargreaves et al. 1988). The mechanical stimulation was repeated 3 times at an interval of 5 min for each test and the mean calculated.

2.8. Sciatic nerve acquisition and tissue preparation

Each rat was deeply anaesthetized with Urothane (500 mg/ml, i.p., 300 g/ml) and were sacrificed on day 22 (D22), day 40 (D40). Under aseptic conditions, skin was cut to expose the left sciatic nerve, proximal to the trifurcation (about 1 cm), before the four ligatures in the CCI animals were removed and immediately frozen in liquid nitrogen and stored at −80℃ for later homogenization and the cytokine expression assay.

Ice-cold homogenization buffer was added (300 µl/sciatica). The homogenization buffer was freshly prepared by adding protease inhibitor (P 8340 cocktail Sigma) to T-PER™ Tissue Protein Extraction Reagent (Pierce Chemical Co., USA) prior to tissue lysis. After adding the buffer, a homogenization probe (Tissue Tearor, Polytron, Biospec Products, Inc.) was applied for 20 s on ice at 21000 RPM. Then the homogenized samples were centrifuged for 40 min at a speed of 13,000 rpm at 4 °C, stored at −80 °C and used subsequently for protein quantification. The protein concentration in the supernatant was quantified using the Lowry protein assay. Samples were pipetted as duplicates (1 µl/50µl/well) in a 96 well microtiter plate (Costar). Each plate was inserted into a plate reader (Molecular Device Spec 383) to read the optical density of each well at an absorbance of 750 nm. Data were analyzed using Ascent Software for iEMS Reader.

2.9. Cytokine analysis

Tumor necrosis factor-alpha (TNF-α) and Interleukin-1 beta (IL-1β) concentrations in the supernatants will be determined using the DuoSet® ELISA Development kit (R&D Systems, Minneapolis,MN, USA). All experimental procedures were in accordance with the instructions.

Plates were singly 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 four-parameter logistics curve-fit. Data were expressed in pg /mg protein of duplicate samples.

2.10. HSP72 analysis

Protein samples (30 μg/lane) were separated on a 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) with a constant voltage of 75V. Electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, 0.45μm pore size) with a transfer apparatus (Bio-rad). PVDF membranes were incubated in 5% milk in TBS buffer. The membrane was blocked in TBS (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20, pH7.5) containing 5% skim milk (Difco, Detroit, MI) for 1 h. Mouse monoclonal anti-HSP72 primary antibody (SPA 810; StressGen Biotechnologies, Victoria, British Columbia, Canada) was diluted to 1:500 in antibody binding buffer overnight at 4 ℃. The membrane was then washed three times with TBS (10 min per wash) and was incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz) diluted 500-fold in TBS buffer at 4 ℃. The membrane was washed in TBS buffer for 10 min three times.

Immunodetection for HSP72 was performed using the enhanced chemiluminescence ECL western Blotting luminal Reagent (Santa Cruz, CA, USA) and then the membrane was quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Tokyo, Japan).

2.11 Histopathological examination

2.11.1 Slices preparation

Each rat was deeply anaesthetized with an i.p. injection of Urethane (500 mg/ml, i.p., 300 g/ml) and were sacrificed on day 40 by exsanguination with the aid of ice-cold heparin-saline (15 IU/L) perfusion and 10% formaldehyde solution (Mallinckrode chemicals). The sciatic nerve were excised and were soaked in 10% formaldehyde solution in aqueous phosphate buffer (Mallinckrode chemicals) for fixation. Subsequently, they accepted a series of processing steps trimming, dehydration, cleaning, infiltration and tissues embedding (Tissue-Tek® TEC™ 5 Tissue Embedding Console System) for paraffin blocks. Then, use the rotary microtome (QP-3268, Histo-Line Laboratories) for paraffin sections. These tissues were sectioned in the longitudinal and cross plane at a thickness of 4 μm on a sliding microtome.

2.11.2 Hematoxylin-Eosin staining

The sciatic nerves of rats were excised and were soaked in 10% paraformaldehyde formalin and covered with wax. Slides (Thremo Scientific) were prepared by deparaffinization with xylene (J.T. Baker) twice for 5 minutes. Then, they were passed through a series of graded alcohols (100%, 95%, 80%, 70% and 50%), 5 minutes each for hydration. The slides were then dyed with hematoxylin (Muto Pure Chemicals Co, LTD) for 1 minute and gently rinsed with water for 1 minute. Subsequently, they were soaked in 0.5% ammonia (Panreac) for 30 seconds. After being gently rinsed with water, the slides were then dyed with eosin for 1 minute. Each slide was then soaked with 95% alcohol, 100% alcohol for 1 minutes each for dehydration. At the end, they were soaked with xylene three times for 5 minutes. Photomicrographs were obtained using Zeiss axiophot microscopes.

2.11.3 Quantification of Wallerian degnereration

The investigators were blinded to the identity of the nerve sections. All sections were coded. All fibers within the whole nerve section that were evaluated were examined under light microscopy at a magnification of 400 X. The investigators (at least three) analyzed/counted the sections and generated a score for nerve degeneration.

Abercrombie & Johnson (1946) indicates a very close correlation between the extents of the increase in size of the nuclear population of a nerve during *Wallerian degeneration*. To determine the changes in the total population, counts of all the nuclei visible in one transverse section, four to ten fields from each of three sections were used, selected at random, of each nerve (Thomas 1948). Mean populations of all nuclei in a complete transverse section of different degenerated nerves, expressed as percentages of the mean populations of undegenerated nerves, the calculation was described as below:

$$\text{Percentage} = \frac{\text{mean number of nuclei of immune cells in CCI}}{\text{mean number of nuclei of immune cells in SO}} \times 100\%$$

2.12. Statistical analysis

The results are presented as mean ± S.E.M. of values. Differences among groups for withdrawal threshold were determined by Student's t test. TNF-α, IL-1β and HSP72 contents were determined using one-way analysis of variance (ANOVA) followed by post hoc Tukey's test for multiple comparisons. The differences of quantification in *Wallerian degnereration* were determined by non-parametric Mann-Whitney U test. Statistical calculations were performed using SPSS for Windows (version 15.0, SPSS, Inc, Chicago, IL, USA). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Body weight

No autotomy was observed in this study. The development of body weight demonstrated in Figure 1. SO compared to control group had no significant difference (Fig. 1AB). CCI compared to SO or control group showed significant body weight loss (Fig. 1AB). The body weight in SOTE or SOSE group compared to SO group demonstrated significant body weight loss (Fig. 1AB). CCI rats with exercise training (CCISE or CCITE group) compared to CCI group had no significant difference in body weight (Fig. 1AB). Furthermore, we also observed animals' grooming, sleep wake cycles and social interaction with other rats in the cage were not obviously affected (data not shown).

3.2. Thermal hyperalgesia and mechanical allodynia

In control, sham operated, sham operated with swimming or treadmill exercise groups, rats showed no thermal hyperalgesia and mechanical allodynia until day 39 after CCI-treatment (Fig. 2). As expected, all the CCI rats developed qualitative signs indicated a significant decrease in thermal withdrawal latency and tactile allodynia withdrawal pressure of the ipsilateral hindpaw after nerve injury, as compared to sham operated animals (Figs. 3 and 4). Both of heat and mechanical pains were clearly present after 24 hrs to the surgery, reached a peak on D7. We also compared CCISE with CCITE of the tendency towards resolution after 39 days exercises training (swimming and treadmill), indicating significant changed in thermal withdrawal latency and paw withdrawal pressure (data not shown).

The time courses of thermal hyperalgesia and mechanical allodynia after 39 days exercise

training (swimming/day and treadmill/weekday) is shown in Figure 2 and Figure 3, respectively. Three days after injury, rats developed a significant decrease in thermal withdrawal latency only at the paw ipsilateral to the injury, as compared to sham-operated animals; rats also developed tactile allodynia to normally innocuous stimulation with von Frey filament. Three days after injury, thermal hyperalgesia attenuated significantly in neuropathic rats treated with treadmill (8.79±0.29 g, $P< 0.01$, n=9, Fig. 3A) exercises and swimming (11.24±1.09 g, $P< 0.05$, n=8, Fig. 4A), while the mechanical allodynia had no significantly changed (Figs. 3B and 4B), as compared to CCI rats. In addition, 21 days after injury, for heat sensitivity, the thermal withdrawal latency of the neuropathic rat treated with treadmill (10.49±0.55 g, $P< 0.05$, Fig. 3A) exercises and swimming (11.53±0.87 g, $P< 0.05$, Fig. 4A); for mechanical sensitivity, the paw withdrawal pressure of the neuropathic rat treated with treadmill (16.41±0.58 g, $P< 0.01$, Fig. 3B) exercises and swimming (16.15±0.48 g, $P< 0.01$, Fig. 4B). After prolonged exercise treatment, there is no significant treatment effect on neuropathic rats except for mechanical allodynia in treadmill (day 28, $P< 0.05$; day 35, $P< 0.05$, Fig. 3B) exercise rats.

3.3. Cytokine expression

Figure 5 depicts the levels of TNF-α and IL-1β in sciatic nerve of CCI, CCISE, and CCITE rats after 21 days exercise training. The expression of TNF-α decrease in CCITE (59.38±3.74 pg/mg protein, $P< 0.05$) rats compared to CCI (75.25±2.13 pg/mg protein) group, not obviously changed in CCISE after 21 days exercise training (Fig.5A). The expression of IL-1β decrease in CCITE (69.25±8.76 pg/mg protein, $P< 0.01$) rats compared to CCI (162.92±19.83 pg/mg protein) group after 21 days exercise training, as shown in Figure 5B. In addition, the IL-1β expression in sciatic nerve

decreased in CCISE (92.25±24.03 pg/mg protein, P<0.05) compared to CCI.

Figure 6 demonstrates the levels of TNF-α and IL-1β in sciatic nerve of CCI, CCISE, and CCITE rats after 39 days exercise training or non-training. The level of TNF-α in sciatic nerve significantly decrease in CCITE (48.39±2.93 pg/mg protein, P< 0.001) and CCISE (67.17±8.59 pg/mg protein, P<0.05) rats after 39 days exercise, compared to CCI (98.65±11.36 pg/mg protein) rats. In addition, the expression of IL-1β decrease in CCITE (130.43±2.98 pg/mg protein, P< 0.05) rats compared to CCI (154.14±2.58 pg/mg protein) group after 39 days exercise training, as shown in Figure 6B.

3.4. HSP72 expression

Figure 7 depicts the expression of HSP72 in sciatic nerve after CCI in different groups. It can be seen that the levels of HSP72 is significantly increased in sciatic nerve after 3 weeks exercise training program (Fig. 7A). The level of HSP72 increased to 3.15 ± 0.77 (P < 0.05, n=5) in CCITE rats after 3 weeks treadmill exercise training. However, the level of HSP72 increased insignificantly (1.92 ± 0.19, n=5) after 3 weeks swimming exercise training. After exercise training 39 days, the level of HSP72 significantly increased in CCTE group compared to CCI group. After exercise training 39 days, the level of HSP72 significantly increased in CCTE group compared to CCI group, about 3.9- fold (P < 0.05, n=4) as shown in Figure 7B. In addition, the level of HSP72 increased insignificantly (0.8- fold, n=4) after swimming exercise training 39 days.

3.5. Histopathological examination

Histological appearance was essentially normal for all sham operated groups, with a similar

distribution of small and large diameter nerve fibers, blood vessels and immune cells into one or more fascicles, each surrounded by a well-defined perineurium, and a regular proportion between myelin sheath thickness and fiber diameter as shown in Figure 8AE for comparison. Axonal degenerations were evident in the sciatic nerve in CCI, CCISE and CCITE rats on D22 and D40, with a large amount of myelin, cell debris and immune cells as well as *Wallerian degnereration* of nerve fibers (Fig. 8). After exercise training 21 days and 39 days, the extent of degeneration significantly attenuated in CCTE (fig. 8CG) and CCISE (fig. 8DH) rats compared to CCI rats (fig. 8BF). Figure 8I shows the mean number of nuclei in a complete transverse section of sciatic nerve at different times (day 21 and day 40), expressed as a percentage of the mean number of nuclei in degenerated nerves.

4. Discussion

We found that swimming and treadmill exercises retarded peripheral neuropathic pain and protect nerve damage following chronic constriction injury of sciatic nerve in rats. Swimming and treadmill exercise decreased TNF-α and IL-1β expression and increased HSP72 expression in sciatic nerve after CCI-treatment.

In this study, rats with exercise (swimming or treadmill) showed body weight loss compared with rats without exercise. However, exercise did not affect the curves of body weight increases after CCI-treatment (Fig. 1AB). We thought that rats suffered from stress (e.g., exercise and CCI) and maintained similar curves of body weight increases.

Regardless of swimming or treadmill exercise, they retarded the decreasing curves of time courses of hyperalgesia and allodynia. CCI rat with swimming or treadmill exercise attenuated thermal hyperalgesia and mechanical allodynia significantly, when compared with CCI rats with exercise 21 days after CCI-treatment (Figs. 3 and 4). These results are agreed with previous studies, which reported swimming exercise (Kuphal et al. 2007) attenuated peripheral neuropathic pain in rats. Treadmill and swimming exercise ameliorated spinal cord injury-induced allodynia and restored normal sensation after spinal cord contusion in rats (Hutchinson et al. 2004).

Neuropathic pain exhibit a series of anatomical, morphological and functional changes occur following damage to the peripheral nerve injury. Evidence has been presented that neuropathic pain consequent peripheral nerve injury is associated with local inflammation and overexpression of inflammation cytokines (Martucci et al. 2008). The present results are consistent with several

previous studies in partly. Previous studies have demonstrated that CCI induce activity in axons became hypersensitivity and enhanced transmission retrogradely to cell bodies in the dorsal root ganglia and spinal cord with subsequently released some mediators. For example, these mediators were able to activate the microglia cell via specific receptors and induce phosphorylation of p38 mitogen-activated protein kinase in spinal cord, where they may alter gene expression of the neurons (Tsuda et al. 2004; Song et al. 2005; Zhang et al. 2005; Burnstock 2006; Inoue 2006; Gu et al. 2008). However, the hyperactive microglia result in the release of bioactive substances, including cytokines, prostaglandin E2 and excitatory amino acids (such as glutamate and aspartate) that alter the responses of dorsal horn cells and maintain the neuropathic pain states (Campbell and Meyer 2006; Inoue 2006). Our results showed that treadmill or swimming exercise training could attenuate TNF-α and IL-1β expression 21 day after chronic constriction injury. This evidence may provide a reasonable explanation for our experimental results why exercise training could alleviate neuropathic pain following CCI in rats.

In agreement with our results, numerous studies demonstrate that exercise has beneficial effects on chronic disease, presents the neuroprotection, anti-inflammatory effect, and neuropathic pain resolution (Woods et al. 2006; Kuphal et al. 2007). Previous studies have demonstrated that exercise-induced modulation of heat shock factor-1 (HSF-1, a HSPs transcription factor) aggregation, subsequently expression of HSP72 in multiple organs or neurons of rats (Hung et al. 2005; Chen et al. 2007; Noble et al. 2008; Hu et al. 2009). In addition, treatment with BRX-220 (co-inducer of HSPs) on the expression of HSP70 lead to slowly developing analgesic actions to allodynia and

enhancement of recovery processes on rat following L5 spinal nerve ligation (Kalmar et al. 2003). Moreover, the increase in HSPs expression can decrease the production of the proinflammatory cytokines has been proved (Saleh et al. 2000). Our results showed 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 sciatic nerve of rats, subsequently improved CCI-induced neuropathic pain. We suggest that the protective effect of HSP72 observed in this study is induced by the exercise mediated aggregation of HSF-1, consequently promoting HSP72 generation in the CCI rat. Although we do not provide direct evidence of the action mechanism of HSP72 attenuated proinflammatory cytokines expression in this study, accumulated evidences showed that HSPs can decrease the production of the proinflammatory cytokines (Saleh et al. 2000). However, these observations on thermal hyperalgesia, mechanical allodynia, and HSP70 are, at present, merely co-incident.

Our neuropathological findings showed serious *Wallerian degeneration*, and axonal, and myelin damage after CCI. Previous studies indicates that *Wallerian degeneration* leads to release of cytokines or other molecules from denervated Schwann cells, mast cells or other cell types that supports the concept that inputs from the peripheral nerve play ongoing critical role with regard to neuropathic pain (Lancelotta et al. 2003). In addition, axonal demyelination and degeneration were similar to those previous studies (Chen et al. 1992; Kato et al. 2002; Mazzer et al. 2008). Conversely, the level of degeneration was decreased by exercise training in the present study. Moreover, regeneration provides reinnervation of these cells and is associated with decreasing hyperalgesia

(Lancelotta et al. 2003). In this study, exercise training may reduce *Wallerian degeneration* which leads to release of cytokines (TNF-α and IL-1β) and increase regeneration which decreased hyperalgesia.

However, there is a limitation of this study. Treadmill and swimming exercise trainings in this study are forced, whereas they in humans are voluntary (Kuphal et al. 2007). Therefore, we must be careful when the findings in this study translate rodent therapeutic strategy to human cases.

5. Conclusions

Our study demonstrated that swimming and treadmill exercise partially ameliorates thermal hyperalgesia, mechanical allodynia, TNF-α and IL-1β expression in sciatic nerve. Treadmill exercise, but not swimming exercise, increased HSP72 expression in sciatic nerve of CCI with exercise rats, compared with CCI without exercise rats. The behavioral improvement with daily exercise treatment may suggest a progressive analgesic action. These results of this study also indicated that treatment with exercise can be beneficial in *Wallerian degeneration*, axonal and myelin damage of sciatic nerve. On the basis of our results we suggest that exercise might represent a potential therapeutic strategy for CCI-induced peripheral neuropathy.

Further studies are required to examine the possible mechanisms of activation, and to investigate the biological actions of HSP72 increased expressions responses to varying types of exercise training.

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Table 1 Graded Swimming Exercise Protocols

Day(s)	*Exercise Period (min)*	*Rest Period (min)*	*Sessions*	*Total Exercise Duration (min)*
-2~1	10	15	9	90
2	15	15	6	90
3	30	15	3	90
4	45	15	2	90
5	60,30	15	2	90
6	75,15	15	2	90
7-39	90	0	1	90

All swimming animals accommodate the water depth and temperature two days before surgery. Chronic constriction injury and sham operation were performed on day 0. Behavioral assessments were measured on 1 day before and before surgery, after surgery day 1, 3, 7, 14, 21, 28, 35 and 39.

Table 2 Graded Treadmill Exercise Protocols

Week(s)	*Day(s)*	*Exercise Rate (km/hr)*	*Total Exercise Duration (min)*
1	day -2 to 4	1.2	15/ 30
2	day 5 to 11	1.8	30
3	day 12 to 18	1.8	60
4	day 19 to 25	1.8	60
5	day 26 to 32	1.8	60
6	day 33 to 39	1.8	60

The rats were run on a treadmill 5 days a week for 6 weeks. On the first week, all rats acclimatized the track and ran for 15 min at 1.2 km/hr, 0% slope for 3 days. The duration and intensity of the exercise were increased progressively. Chronic constriction injury and sham operation were performed on day 0. Behavioral assessments were measured on 1 day before and before surgery, after surgery day 1, 3, 7, 14, 21, 28, 35 and 39.

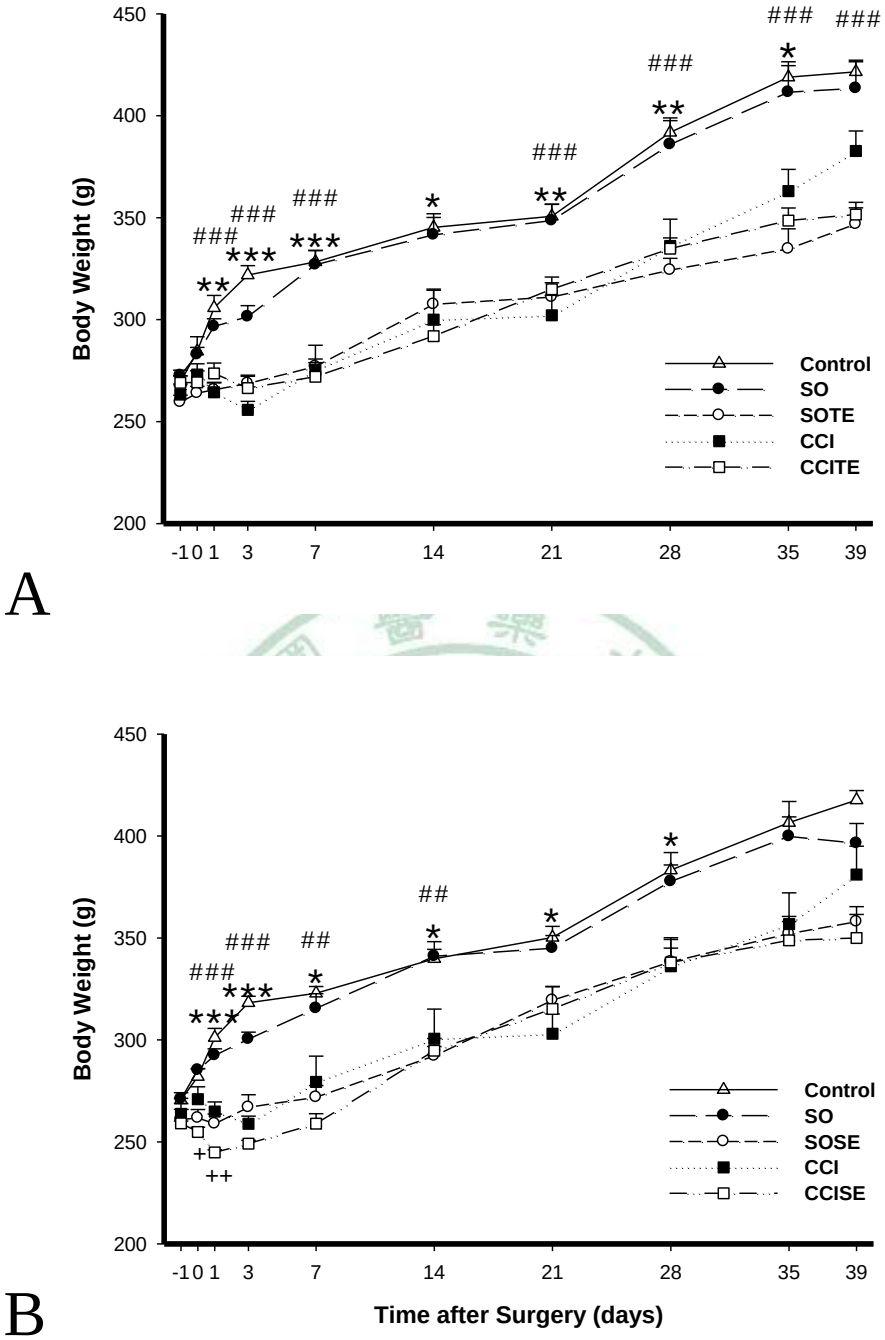


Fig. 1. Body weight change of treadmill (A) and swimming (B) exercised or non-exercised training on control, sham operated (SO) and neuropathic rats (CCI). Data represented as mean ± S.E.M of 8 to 10 rats per group. Symbols (*,**,***) indicate $P < 0.05$, $P < 0.01$, $P < 0.001$ when SO compare to CCI; ($^{\#}$.$^{\#\#}$,$^{\#\#\#}$) indicated $P < 0.05$, $P < 0.01$, $P < 0.001$ when SO compare to SOTE or SOSE; ($^{+}$,$^{++}$) indicated $P < 0.05$, $P < 0.01$ when CCI compare to CCISE. (one-way ANOVA followed by post hoc Tukey's test) (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)

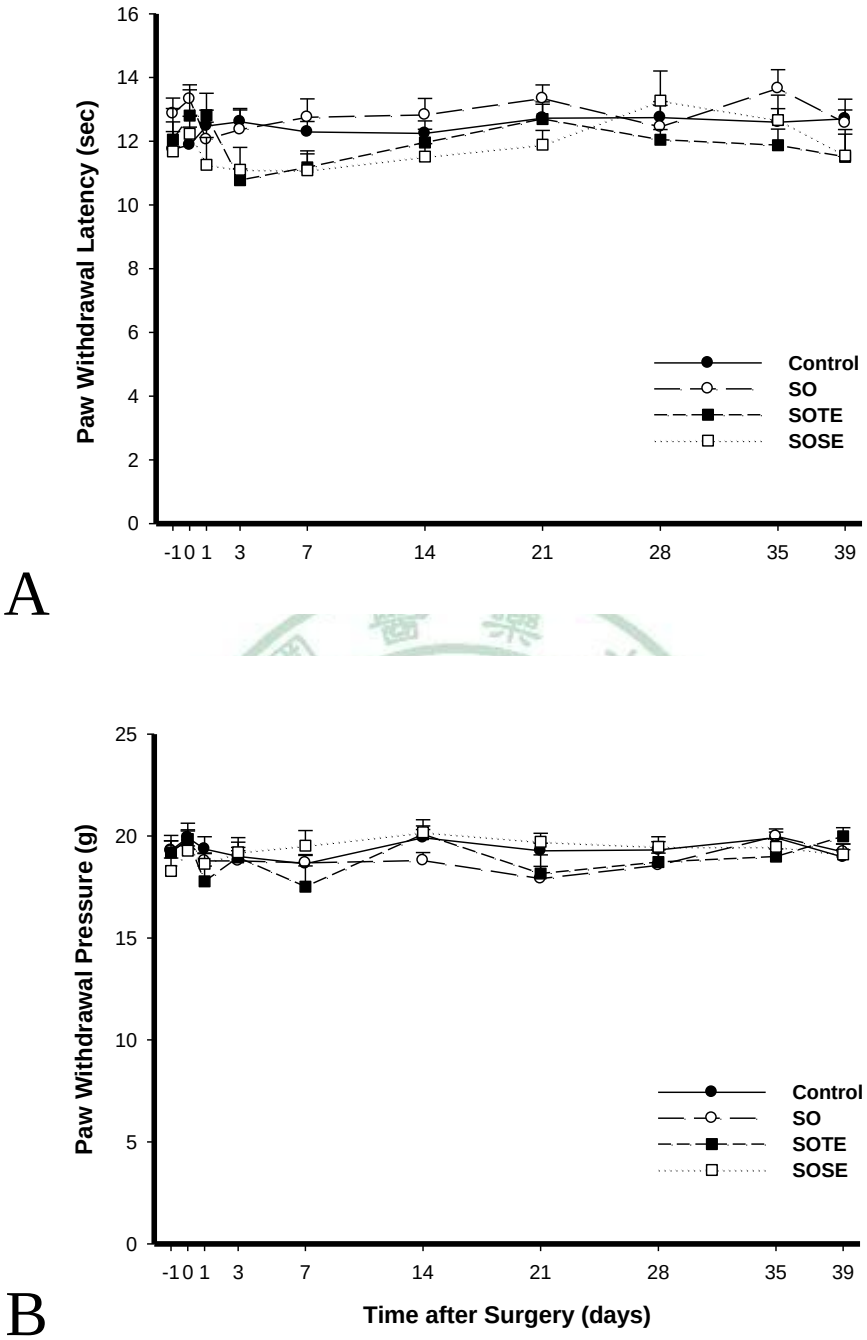
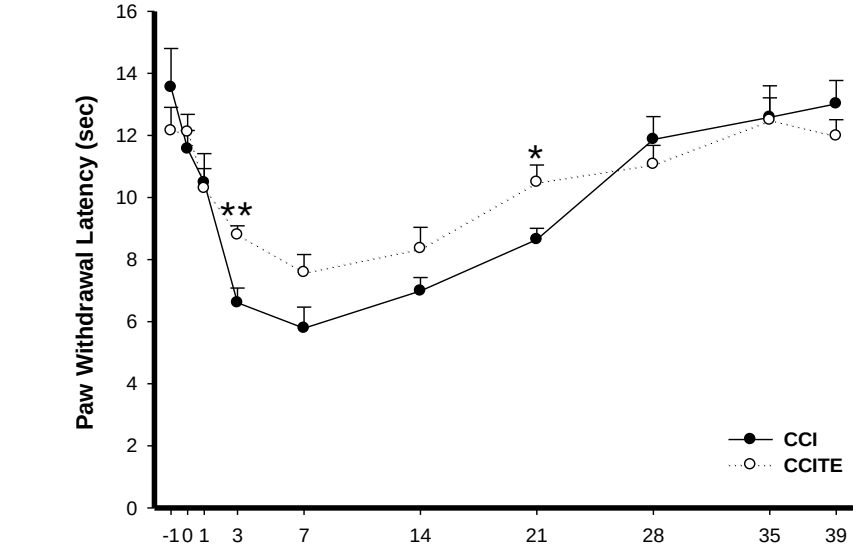


Fig. 2. Time courses of thermal hyperalgesia (A) and mechanical allodynia (B) in control, sham operated (SO) rats and sham operated exercise (SOTE or SOSE) rats. (see Fig. 1 abbreviations). The paw withdrawal latency (s) and pressure (g) to heat and mechanical stimulation were no significant differences among these groups compare to control, respectively. Data represented as mean ± S.E.M of 8 to 10 rats per group. (one-way ANOVA followed by post hoc Tukey's test)



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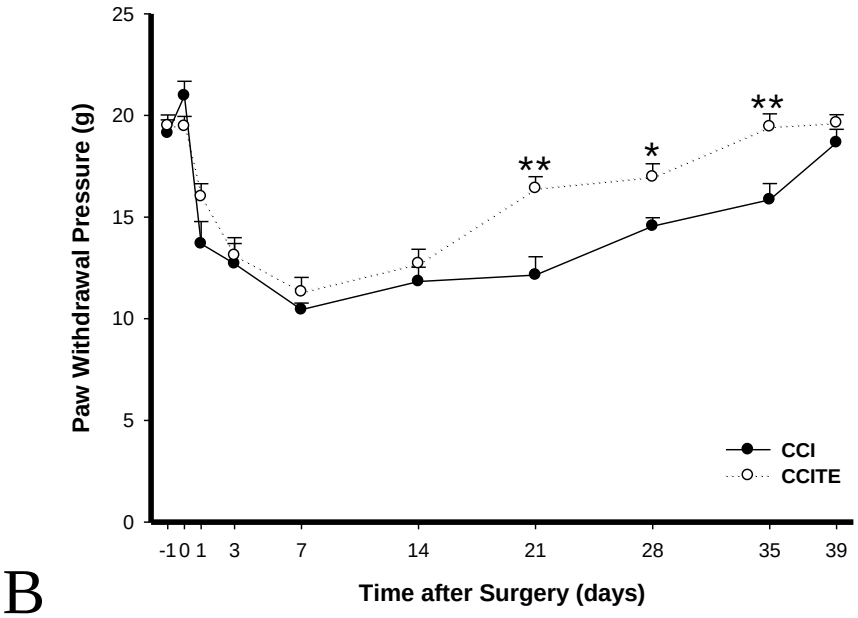
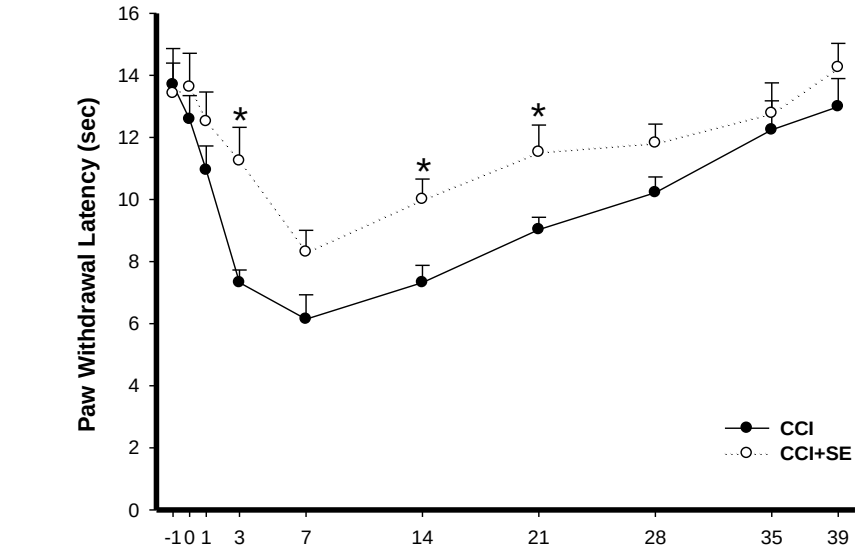


B

Fig. 3. Time courses of thermal hyperalgesia (A) and mechanical allodynia (B) in CCI and CCITE rats. (see Fig. 1 abbreviations). Data represented as mean ± S.E.M of 6 to 8 rats per group. * $P < 0.05$, ** $P < 0.01$, as compared to CCI (Student's t test)



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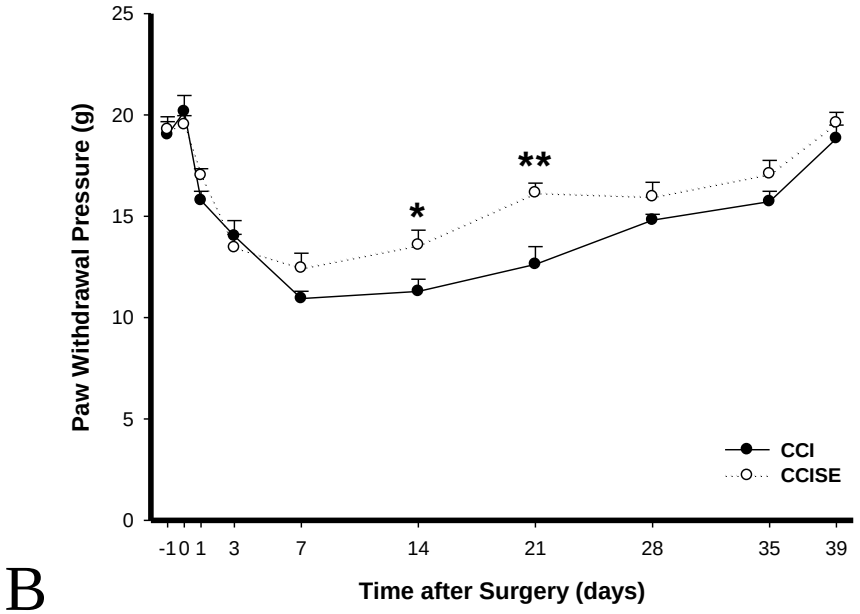


B

Fig. 4. Time courses of thermal hyperalgesia (A) and mechanical allodynia (B) in CCI and CCISE rats. (see Fig. 1 abbreviations). Data represented as mean ± S.E.M of 6 to 8 rats per group. * $P < 0.05$ as compared to CCI (Student's t test)

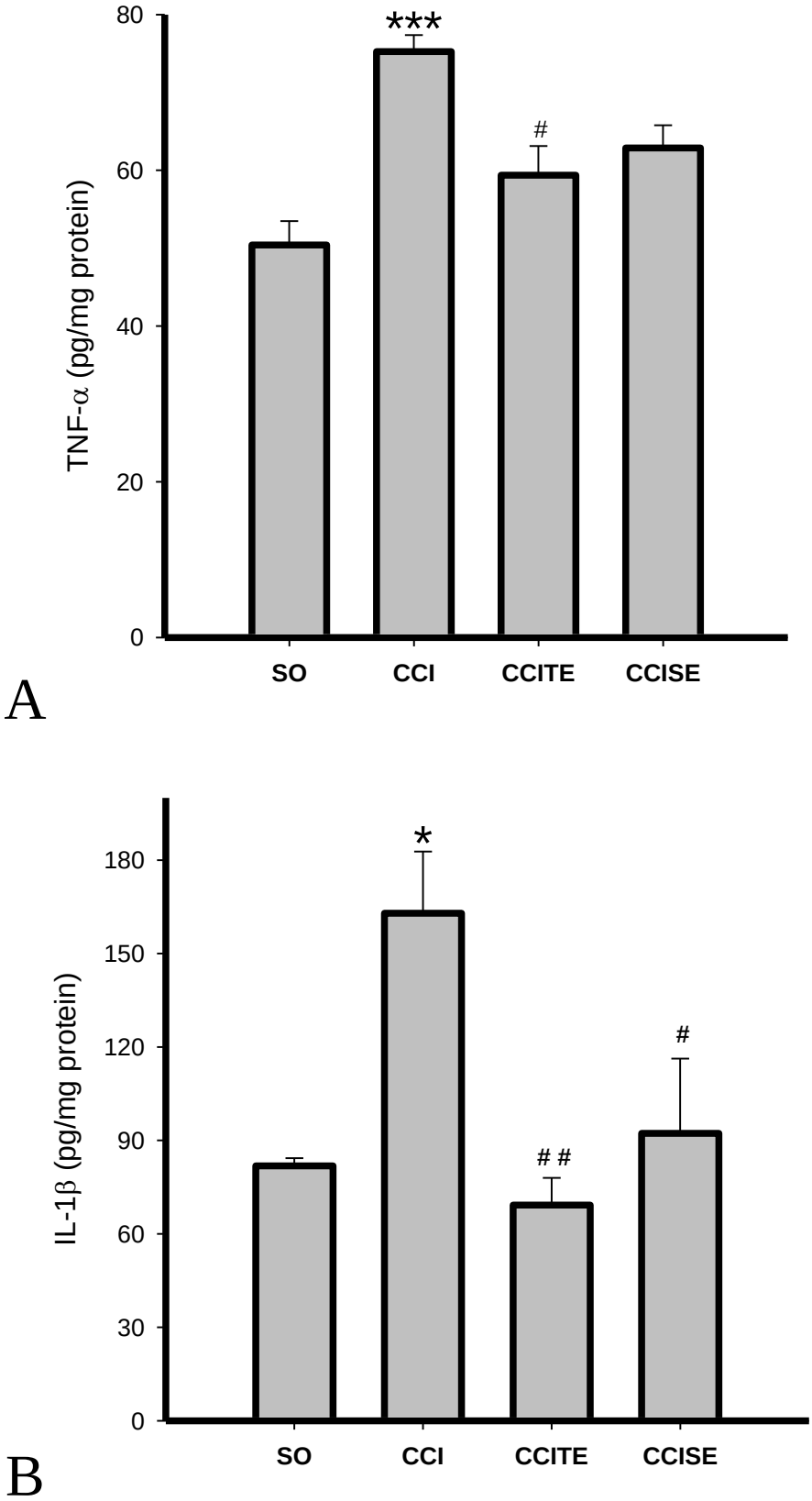
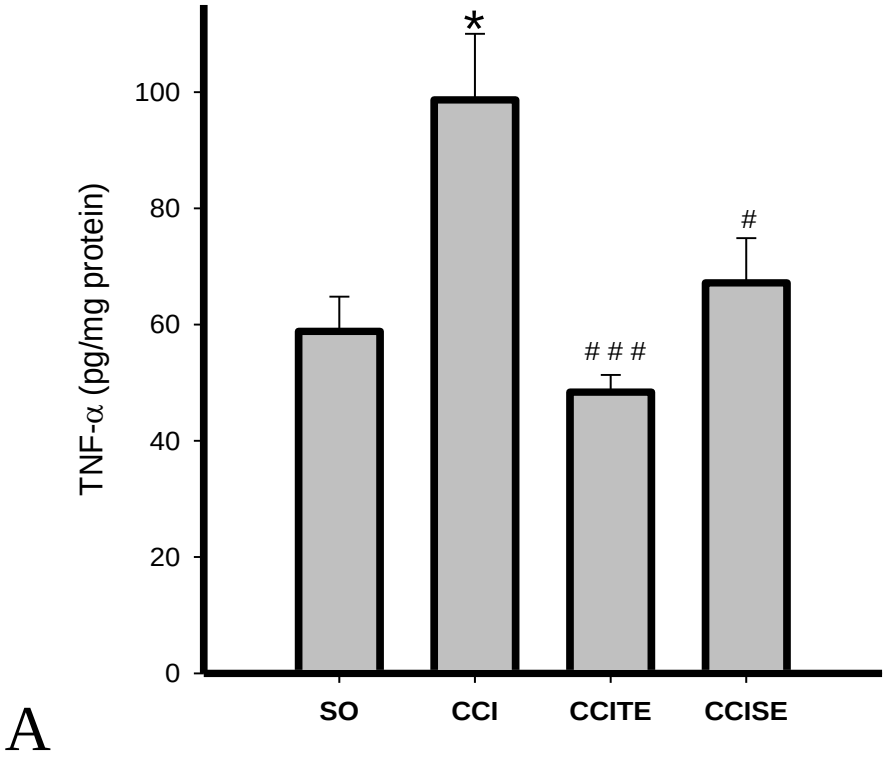


Fig. 5. The level of TNF-α (A) and IL-1β (B) in sciatic nerve on D22 in different groups of rats: SO, CCI, CCITE, and CCISE. (see Fig. 1 abbreviations). The values represented mean ± S.E.M. of 5 rats per group. * $P < 0.05$, *** $P < 0.001$ as compared to SO; $^{\#}P<0.05$, $^{\#\#}P<0.01$ as compared to CCI (one-way ANOVA followed by post hoc Tukey's test)



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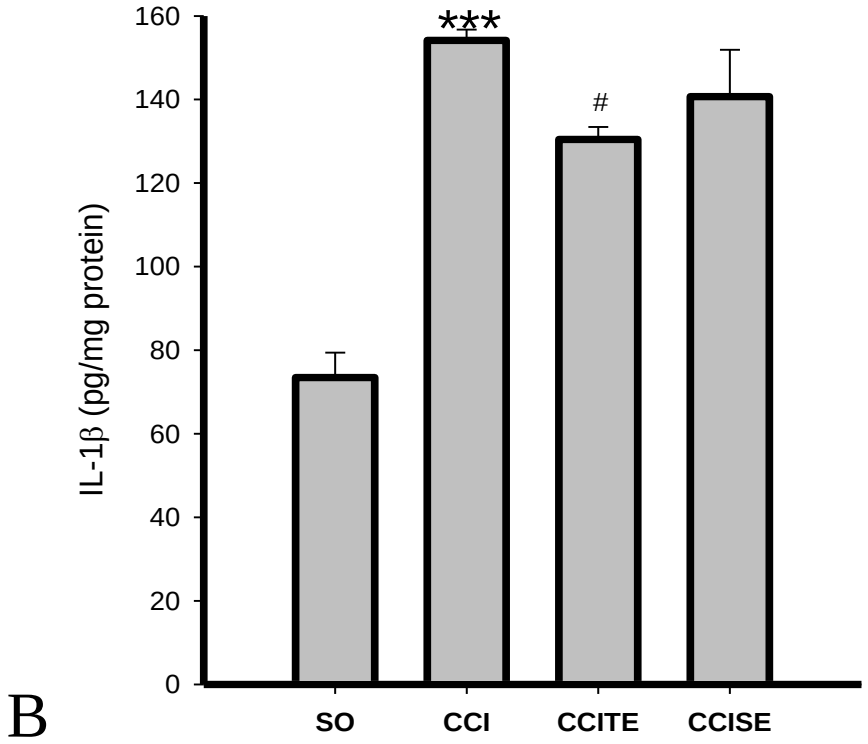

B

Fig. 6. The level of TNF-α (A) and IL-1β (B) in sciatic nerve on D40 in different groups of rats: SO, CCI, CCITE, and CCISE. (see Fig. 1 abbreviations). The values represented mean ± S.E.M of 5 rats per group. * $P<0.05$,*** $P<0.001$ versus SO; $^{\#}$ $P<0.05$, $^{\#\#\#}$ $P<0.001$ versus CCI (one-way ANOVA followed by post hoc Tukey's test)

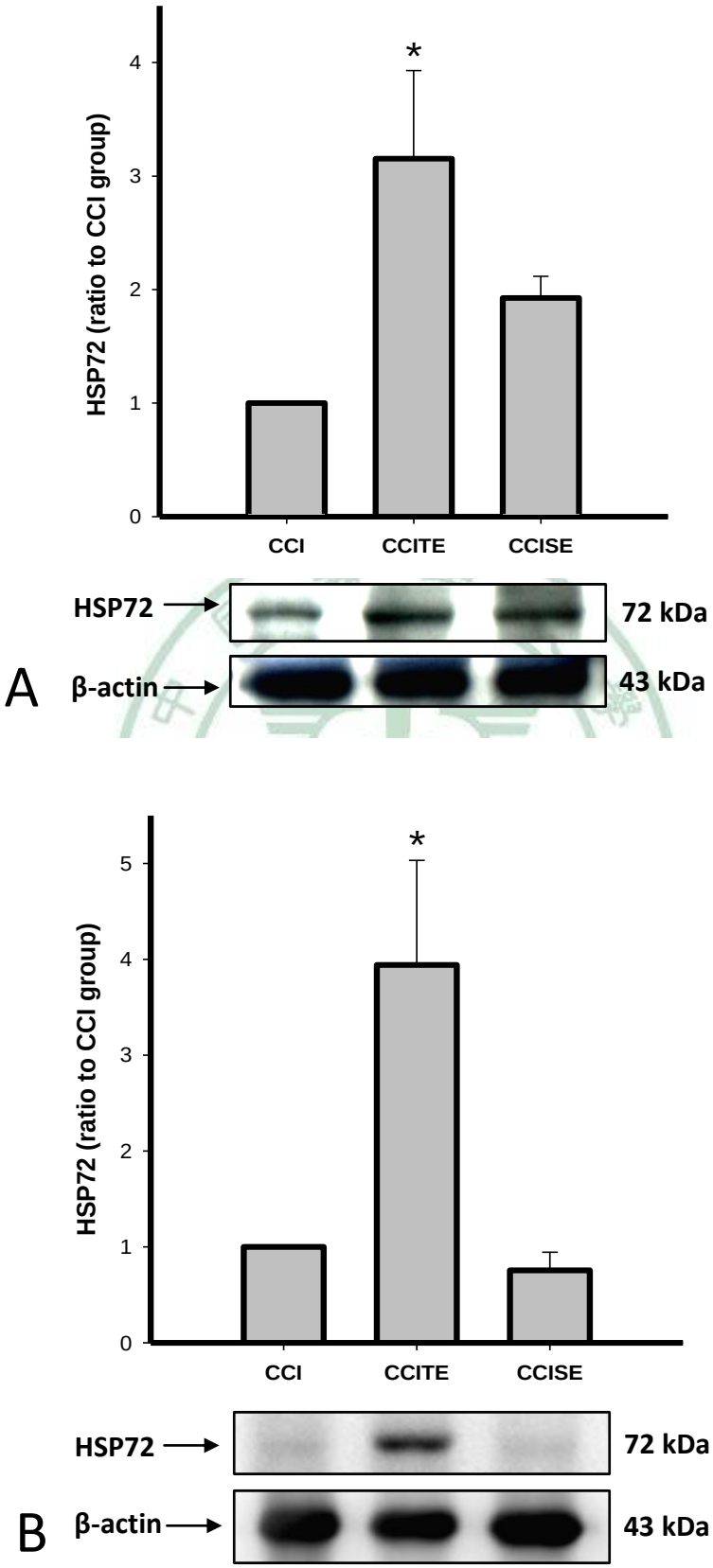

Fig. 7. The level of HSP72 in sciatic nerve on D22 (A) and D40 (B) in different groups of rats: CCI, CCITE and CCISE. (see Fig. 1 abbreviations). The values represented mean ± S.E.M. of 5 rats per group. * $P<0.05$ as compared to CCI. (one-way ANOVA followed by post hoc Tukey's test)

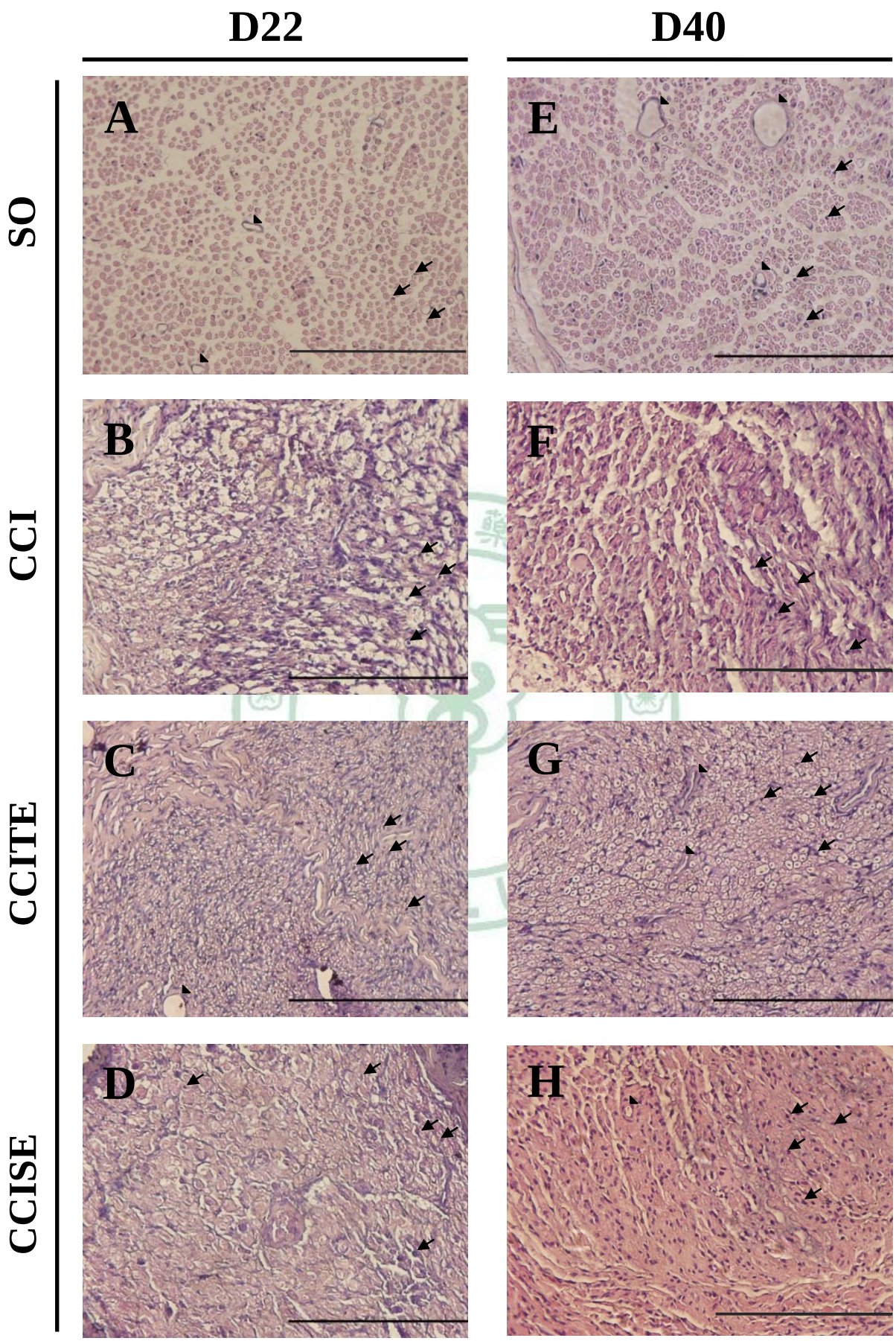

Fig. 8. The histopathological examination of the sciatic nerve on D22 and D40 in different groups of rats: SO (A,E), CCI (B,F), CCITE (C,G) and CCISE (D,F) groups. H&E stain 400X. (Scale bar = 200μm) Arrow (➶) indicates nuclei of immune cells; arrowhead (◣) indicates blood vessels.

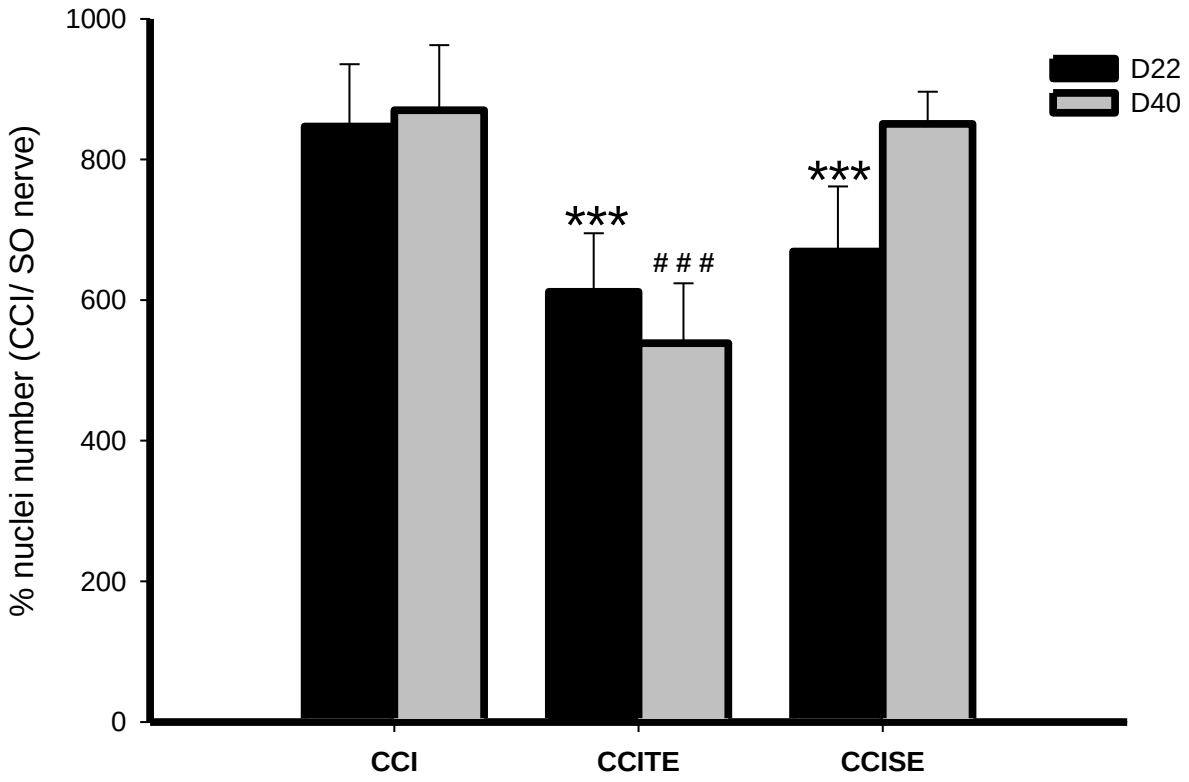

Fig. 9. Quantitative histopathology of *Wallerian degnereration* in the sciatic nerve sectioned transversely at 4μm. The values represented mean ± S.E.M. of 3 sections per group. (Non-parametric Mann-Whitney U test) *** $P < 0.001$ as compared to CCI on D22; $^{###}$ $P < 0.001$ as compared to CCI on D40