

Original Article

Combination therapy with extracorporeal shock wave and melatonin markedly attenuated neuropathic pain in rat

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Abstract: This study tested the hypothesis that combination therapy using extracorporeal shock wave (ECSW)-melatonin (Mel) was superior to either alone at ameliorating neuropathic pain (NP). NP was induced by chronic constriction injury (CCI) to the left sciatic nerve in rats. Animals were categorized into sham control (group 1), CCI only (group 2), CCI-ECSW (group 3), CCI-Mel (group 4) and CCI-ECSW-Mel (group 5). By days 2 and 8 after CCI, the mechanical paw withdrawal threshold (MPWT)/thermal paw withdrawal latency (TPWL) were highest in group 2, lowest in group 1, significantly lower in group 5 than in groups 3 and 4 (all $p < 0.0001$), and not significantly different between groups 3 and 4. The protein expressions of inflammatory (TNF- α /NF- κ B/MMP-9/IL-1 β /GFAP/ox42), oxidative-stress (NOX-1/NOX-2/NOX-4/oxidized protein), DNA/mitochondrial-damaged (γ -H2AX/cytosolic mitochondria), apoptotic (cleaved caspase-3/PARP), and MAPK family biomarkers (p-P38/p-JNK/p-ERK1/2) in dorsal root ganglia and spinal dorsal horn expressed a similar pattern of MPWT/TPWL among the five groups, except for significantly higher in group 4 than in group 3 (all $p < 0.0001$). The protein expressions of Nav.1.3, Nav.1.8 and Nav.1.9 in sciatic nerve displayed an identical pattern to inflammation among the five groups (all $p < 0.001$). Pain facilitated cellular expressions (p-P38+/peripherin+ cells, P38+/NF200+ cells) displayed an identical pattern to inflammation among the five groups (all $p < 0.0001$). In conclusion, ECSW-Mel combination therapy markedly ameliorated NP induced by CCI.

Keywords: Neuropathic pain, chronic constriction injury, inflammation, oxidative stress, extracorporeal shock wave, melatonin

Introduction

Neuropathic pain (NP) disorders are common and have significant psychological and functional effects on individuals, leading to wider socioeconomic impact [1, 2]. NP typically results from lesions or diseases involving the peripheral nerve, dorsal root ganglion, dorsal root, or central nervous system [3]. Despite advanced pharmaceuticals for NP such as tricyclic antidepressants, anticonvulsants, calcium channel ligands and topical lidocaine [4-6], many patients continue to experience refractory pain. There thus remains a need for a new

safe and effective treatment modality for this unresolved problem.

The hallmarks of NP are peripheral and central sensitization, which arise through various complex pathophysiological mechanisms making the disorder difficult to treat [3]. Studies have consistently identified that persistent inflammation [7-9], oxidative stress [10-12], inflammatory cell infiltration and cytokine production [7-9] in the damaged/inflammatory tissue and organ, play central roles for the initiation and propagation of NP. A management strategy involving (1) anti-inflammation, (2) suppression

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of oxidative stress and (3) relief from pain without significant side effects would thus seem ideal.

Extracorporeal shock wave (ECSW) treatments were originally used to fragment painful body calculi. However, its application for treating soft tissue pain is gaining interest since significant pain relief has been reported even in circumstances where the symptomatic calcium deposits have not successfully been disintegrated [13]. ECSW therapy is known to suppress musculoskeletal pain and tendinopathy or fasciitis, implicating a role in attenuating certain inflammatory and pain disorders [14-16]. We have additionally shown that ECSW therapy possesses anti-inflammatory, anti-oxidative, anti-apoptotic, and neuroprotective capacities in the settings of diabetic neuropathy [17] and ischemic organ dysfunction [18, 19]. Separately, melatonin, an indole hormone secreted mainly by the pineal gland, appears to play important roles in pain relief [20-23], maintenance of cell membrane stability, and enhancing cell survival within stressed environments mainly by reducing cellular susceptibility to oxidative stress and free radical damage, and by suppressing the inflammatory reaction [20, 23-28].

Combining therapies with discrete neuroprotective, anti-inflammatory and antioxidant capabilities may have additive benefits to those already provided by current NP treatments [29]. Combination therapies often have synergistic effects, offering greater than expected benefits for patients with intractable medical conditions. Based on the aforementioned reports [14, 15, 17-21, 23, 25], this experimental study tested the hypothesis that combined therapy using ECSW and melatonin may be superior to either alone for offering antinociceptive effects in a rat model of NP.

Materials and methods

Ethics

All animal experimental procedures were approved by the Institute of Animal Care and Use Committee at Kaohsiung Chang Gung Memorial Hospital (Affidavit of Approval of Animal Use Protocol No. 2015051303) and performed in accordance with the Guide for the Care and Use of Laboratory Animals [The Eighth Edition of the Guide for the Care and Use of Laboratory Animals (NRC 2011)].

Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved animal facility in our hospital with controlled temperature and light cycles (24°C and 12/12 light cycle).

Animal model of neuropathic pain

Left sciatic mononeuropathies in rats were induced using the chronic constriction injury (CCI) procedure as previously described [30]. Under adequate isoflurane anesthesia, the left sciatic nerve was surgically dissected and exposed. Four chromic gut ties (4-0) were used to ligate the nerve at 1 mm intervals loosely enough so that, under microscope inspection, the epineural blood circulation was not obstructed. The incision was then closed in layers and animals recovered from anesthesia. Sham surgery involved dissecting and exposing the sciatic nerve without performing ligations.

Animal grouping and treatment strategy

Pathogen-free, adult male Sprague-Dawley (SD) rats ($n = 40$) weighing 325-350 g (Charles River Technology, BioLASCO Taiwan Co. Ltd., Taiwan) were randomly divided into five groups: group 1 [sham control (SC), i.e., sciatic nerve exposure without ligatures], group 2 (CCI only), group 3 [CCI + ECSW (0.12 mJ/mm², 200 impulses/time at post-CCI 3 h, day 3, and 7, skin surface above the femoral areas)], group 4 [CCI + melatonin (50 mg/kg at post-CCI 3 h and 20 mg/kg at post-CCI 18/48 h, intra-peritoneal), and group 5 (CCI + ECSW + melatonin). ECSW energy and melatonin dosages used for treating CCI-induced neuropathic pain were based on our previous reports [17, 25, 31-33].

Behavioral assessments

To elucidate the impact of ECSW-melatonin therapy on suppressing the neuropathic pain at acute and subacute stages, thermal and mechanical nociceptive thresholds were measured before CCI and on post-CCI days 2 and 8. To assess for thermal hyperalgesia, the animal was placed on a glass plate and radiant heat (Plantar Test Apparatus; UgoBasile, Italy) was applied to the plantar surface of the operated hind paw. The withdrawal latency and duration were recorded, with a minimum value set at 0.1 s and a cut-off latency set at 30 s to avoid paw injury. Each rat was tested three times at an

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interval of 5 min, and mean values were used in the analysis.

To assess for mechanical allodynia, the animal was placed in a chamber and a servo-controlled mechanical stimulus (Dynamic Plantar Aesthesiometer; UgoBasile, Italy) was applied to the plantar surface of the operated hind paw repeatedly at 5-min intervals with increasing punctate pressure until the rat withdrew its paw. A maximal cut-off value was set at 50 g to prevent paw damage. The threshold was tested thrice for each time point and mean values were used in the analysis.

Western blot analysis

The ipsilateral sciatic nerve, L4-L5 dorsal root ganglia (DRGs) and corresponding dorsal horn of the spinal cord from the rats of the sham control and experimental groups were harvested as previously described [25, 33-36]. Equal amounts (50 µg) of protein extracts were loaded and separated by SDS-PAGE using 8-12% acrylamide gradients. After electrophoresis, the separated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences). Nonspecific sites were blocked by incubation of the membrane in blocking buffer [5% nonfat dry milk in T-TBS (TBS containing 0.05% Tween 20)] overnight. The membranes were incubated with the indicated primary antibodies [cleaved caspase 3 (1:1000, Cell Signaling), cleaved poly (ADP-ribose) polymerase (PARP) (1:1000, Cell Signaling), cytosolic cytochrome C (1:2000, Millipore), NADPH oxidase (NOX)-1 (1:2000, Sigma), NOX-2 (1:750, Sigma), NOX-4 (1:1000, Abcam), interleukin (IL)-1β (1:1000, Cell Signaling), tumor necrosis factor (TNF)-α (1:1000, Cell Signaling), nuclear factor (NF)-κB (1:600, Abcam), matrix metalloproteinase (MMP)-9 (1:3000, Abcam), phosphorylated histone H2AX (γ-H2AX) (1/1000, Abcam), phosphorylated (p)-p38 (1:1000, Cell Signaling), p-JNK (1:1000, Abcam), p-ERK1/2 (1:1000, Abcam), Nav 1.3 (1:200, Alomone Labs), Nav 1.8 (1:2000, Abcam), Nav 1.9 (1:200, Alomone Labs) and actin (1:10000, Millipore)] for 1 hour at room temperature. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin IgG (1:2000, Cell Signaling) was used as the secondary antibody for one hour incubation at room temperature. The washing procedure was repeated eight times within an hour, and immu-

noreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) after exposure to Biomax L film (Kodak). For quantification, ECL signals were digitized using Labwork software (UVP).

Immunofluorescent (IF) staining

IF staining proceeded as our previously reported [37, 38]. Rehydrated paraffin sections were first treated with 3% H₂O₂ for 30 minutes and incubated with Immuno-Block reagent (BioSB, Santa Barbara, CA, USA) for 30 minutes at room temperature. Sections were then incubated with primary antibodies specifically against p-p38 (1:500, Gene Tex), NF-200 (7.5 µg, Abcam), and peripherin (1:1000, Abcam). Sections incubated with irrelevant antibodies served as controls. Three sections of DRG specimens were analysed in each rat. For quantification, three randomly selected high power fields (HPFs) were analysed per section. The mean number of positively-stained cells per HPF for each animal was determined across all nine HPFs.

Oxidative stress reaction in lung parenchyma

The procedure for assessing protein expression of oxidative stress has previously been described [17, 25, 39], using the Oxyblot Oxidized Protein Detection Kit (Chemicon S7-150, Billerica, MA, USA). For quantification, ECL signals were digitized using Labwork software (UVP, Waltham, MA, USA).

Statistical analysis

Quantitative data are expressed as mean ± SD. Statistical analysis was performed by ANOVA, followed by Bonferroni multiple-comparison post hoc test. Statistical analysis was also performed using SPSS (SPSS for Windows, version 13; SPSS, IL, U.S.A.). The threshold for statistical significance was considered P<0.05.

Results

Mechanical paw withdrawal threshold (MPWT) and thermal paw withdrawal latency (TPWL) at days 2 and 8 after CCI (Figure 1)

By day 2 after CCI, MPWT was significantly reduced in group 2 (CCI), group 3 (CCI-ECSW), group 4 (CCI-Mel) and group 5 (CCI-ECSW-Mel) compared to group 1 (SC), significantly reduced

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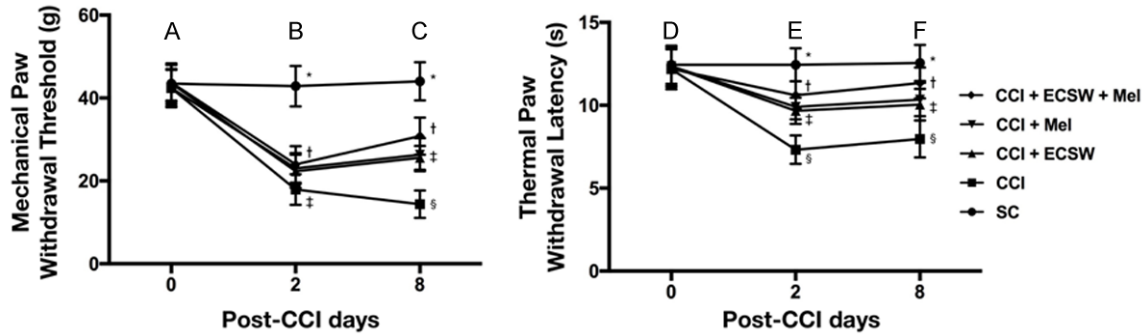


Figure 1. MPWT and TPWL at days 2 and 8 in an experimental model of CCI. Left Panel: A. By day 0, the analytical results of MPWT did not differ among the five groups. B. By day 2, the analytical results of MPWT, * denotes statistical significance vs. other groups with different symbols (†, ‡, §), $p < 0.0001$. The symbol † indicated CCI + ECSW, CCI + Mel and CCI + ECSW + Mel groups. C. By day 8, the analytical results of MPWT, * denotes statistical significance vs. other groups with different symbols (†, ‡, §), $p < 0.0001$. The symbol ‡ indicated CCI + ECSW and CCI + Mel groups. Right Panel: D. By day 0, the analytical results of MPWT did not differ among the five groups. E. By day 2, the analytical results of TPWL, * denotes statistical significance vs. other groups with different symbols (†, ‡, §), $p < 0.0001$. The symbol ‡ indicated CCI + ECSW and CCI + Mel groups. F. By day 8, the analytical results of TPWL, * denotes statistical significance vs. other groups with different symbols (†, ‡, §), $p < 0.0001$. The symbol ‡ indicated CCI + ECSW and CCI + Mel groups. All statistical analyses were performed by one-way ANOVA and Bonferroni multiple comparison post-hoc test ($n = 8$ for each group). Symbols (*, †, ‡, §) indicate statistical significance. MPWT = mechanical paw withdrawal threshold; TPWL = thermal paw withdrawal latency; SC = sham control; CCI = chronic constriction injury; ECSW = extracorporeal shock wave; Mel = melatonin.

in group 2 than in groups 3 to 5, and not significantly different amongst groups 3 to 5. By day 8 after CCI, MPWT was significantly reduced in groups 2 to 5 compared to group 1, significantly reduced in groups 3 and 4 than group 5, more significantly reduced in group 2 than in group 5, but not significantly different between groups 3 and 4. This suggested that combined therapy was superior to either one alone for reducing MPWT.

By day 2 after CCI procedure, TPWL was significantly highest in group 1, lowest in group 2, significantly lower in groups 3 and 4 than in group 5, and not significantly different between groups 3 and 4. By day 8 after CCI procedure, TPWL showed an identical pattern among the five groups.

The protein expressions of sciatic nerve- and L4-5 DRGs-voltage-gated sodium channels and inflammatory biomarkers in L4-5 DRGs by day 8 after CCI (Figure 2)

The protein expressions of Nav.1.3, Nav.1.8 and Nav.1.9, three indicators of voltage-gated sodium channels for sciatic nerve, were lowest in group 1, highest in group 2, significantly higher in groups 3 and 4 than in group 5, and significantly higher in group 4 than in group 3. Additionally, the protein expressions of Nav.1.3,

Nav.1.8 and Nav.1.9, indicators of voltage-gated sodium channels for L4-5 DRGs, exhibited an identical pattern to sciatic nerve among the five groups.

The protein expressions of TNF- α , NF- κ B, MMP-9 and IL-1 β , four inflammatory biomarkers in L4-5 DRGs, were highest in group 2, lowest in group 1, significantly higher in groups 3 and 4 than in group 5, and significantly higher in group 4 than in group 3.

Protein expressions of apoptotic, mitochondrial-damaged, DNA damaged and oxidative stress biomarkers in L4-5 DRGs by day 8 after CCI (Figure 3)

The protein expression of cleaved caspase 3 and cleaved PARP, two indicators of apoptosis, were highest in group 2, lowest in group 1, significantly higher in groups 3 and 4 than in group 5, and significantly higher in group 4 than in group 3. Additionally, the protein expressions of γ -H2AX, an indicator of DNA damage, and cytosolic cytochrome C, an indicator of mitochondrial-damage, exhibited an identical pattern to apoptosis among the five groups.

The protein expressions of NOX-1, NOX-2, NOX-4 and oxidized protein, four indicators of oxidative stress, were highest in group 2, low-

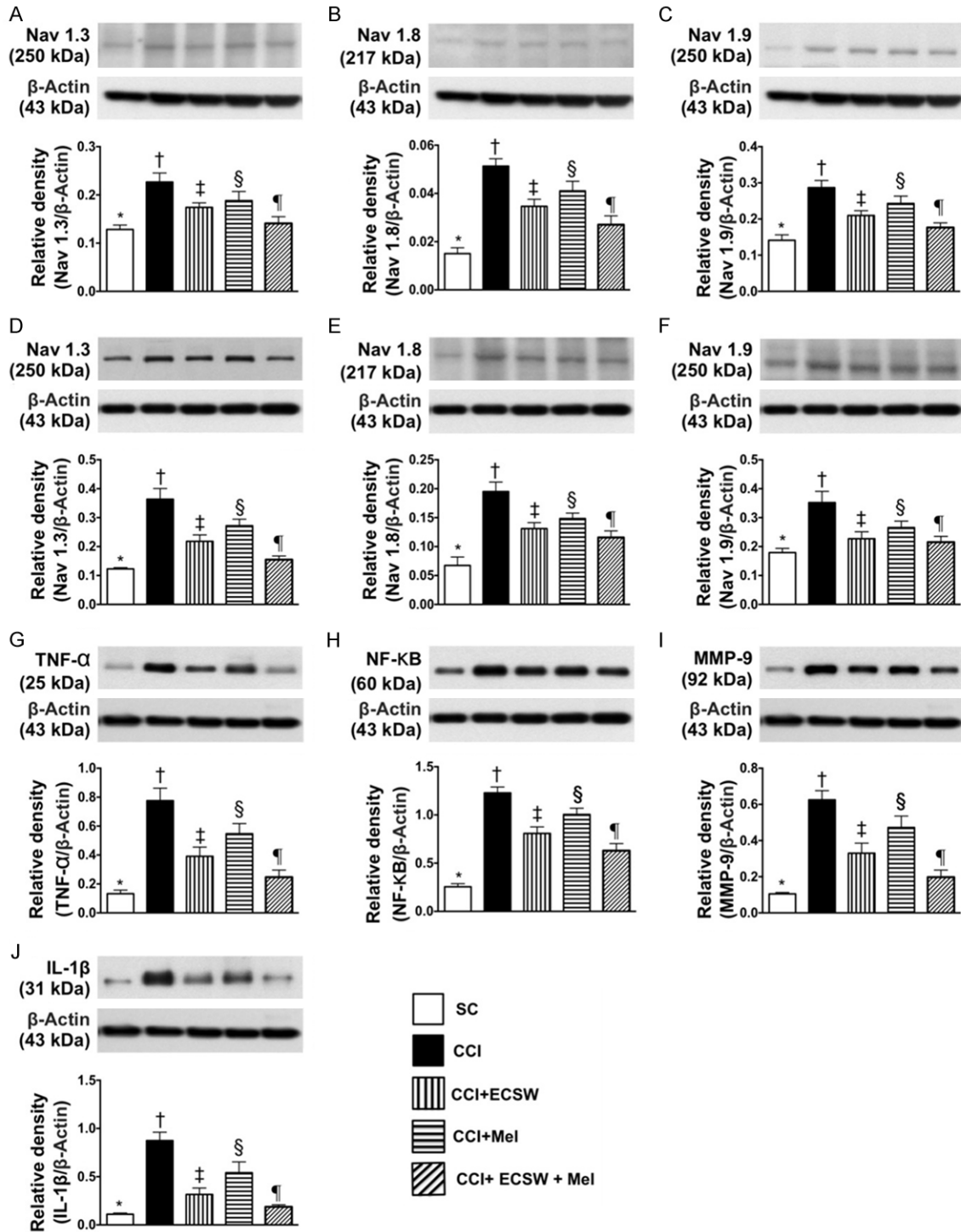


Figure 2. The protein expressions of sciatic nerve- and L4-5 dorsal root ganglions (DRGs)-voltage-gated sodium channels and the protein expressions of inflammatory biomarkers in L4-5 DRGs by day 8 after CCI. A. Protein expression of Nav.1.3 in sciatic nerve, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.001$. B. Protein expression of Nav.1.8 in sciatic nerve, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.001$. C. Protein expression of Nav.1.9 in sciatic nerve, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.001$. D. Protein expression of Nav.1.3 in L4-5 DRGs, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. E. Protein expression of Nav.1.8 in L4-5 DRGs, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.001$. F. Protein expression of Nav.1.9 in L4-5 DRGs, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.001$. G. Protein expressions of tumor necrosis factor (TNF)- α , * denotes sta-

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tistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. H. Protein expression of necrosis factor (NF)- κ B, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. I. Protein expression of matrix metalloproteinase (MMP)-9, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. J. Protein expression of interleukin (IL)-1 β , * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test ($n = 8$ for each group). Symbols (*, †, ‡, §, ¶) indicate significance at the 0.05 level. SC = sham control; CCI = chronic constriction injury; ECSW = extracorporeal shock wave; Mel = melatonin.

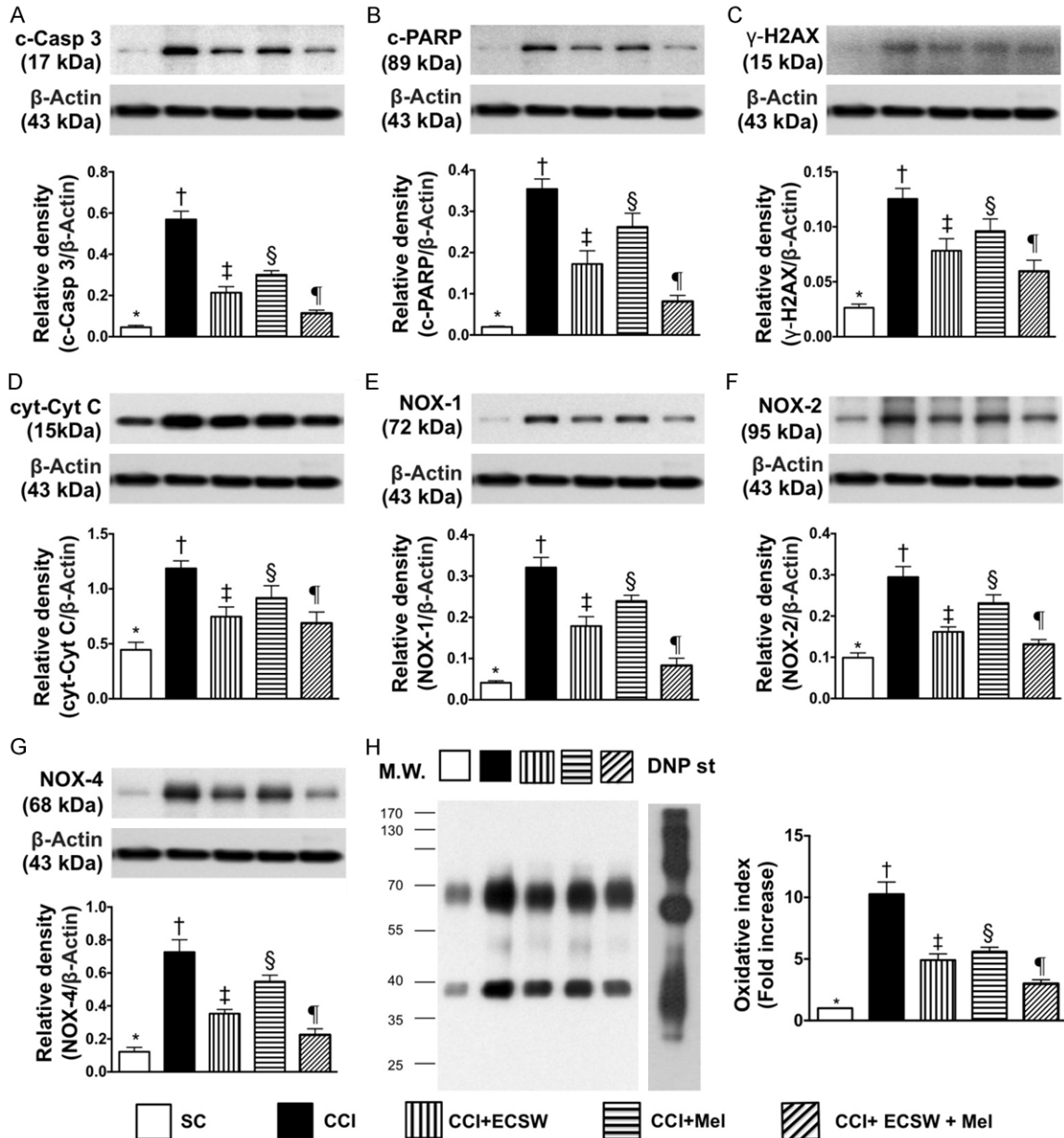


Figure 3. Protein expressions of apoptotic, mitochondrial-damaged, DNA damaged and oxidative-stress biomarkers in L4-5 dorsal root ganglions by day 8 after CCI procedure. A. Protein expression of cleaved caspase 3 (c-Casp 3), * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. B. Protein expression of cleaved poly (ADP-ribose) polymerase (PARP), * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. C. Protein expression of γ -H2AX, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. D. Protein expression of cytosolic cytochrome C (cyt-Cyt C), * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.001$. E. Protein expression of NOX-1, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. F. Protein expression of NOX-2, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$.

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G. Protein expression of NOX-4, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. H. Oxidized protein expression, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. (Note: left and right lanes shown on the upper panel represent protein molecular weight marker and control oxidized protein standard, respectively). M.W = molecular weight; DNP = 1-3 dinitrophenylhydrazine. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test ($n = 8$ for each group). Symbols (*, †, ‡, §, ¶) indicate significance at the 0.05 level. SC = sham control; CCI = chronic constriction injury; ECSW = extracorporeal shock wave; Mel = melatonin.

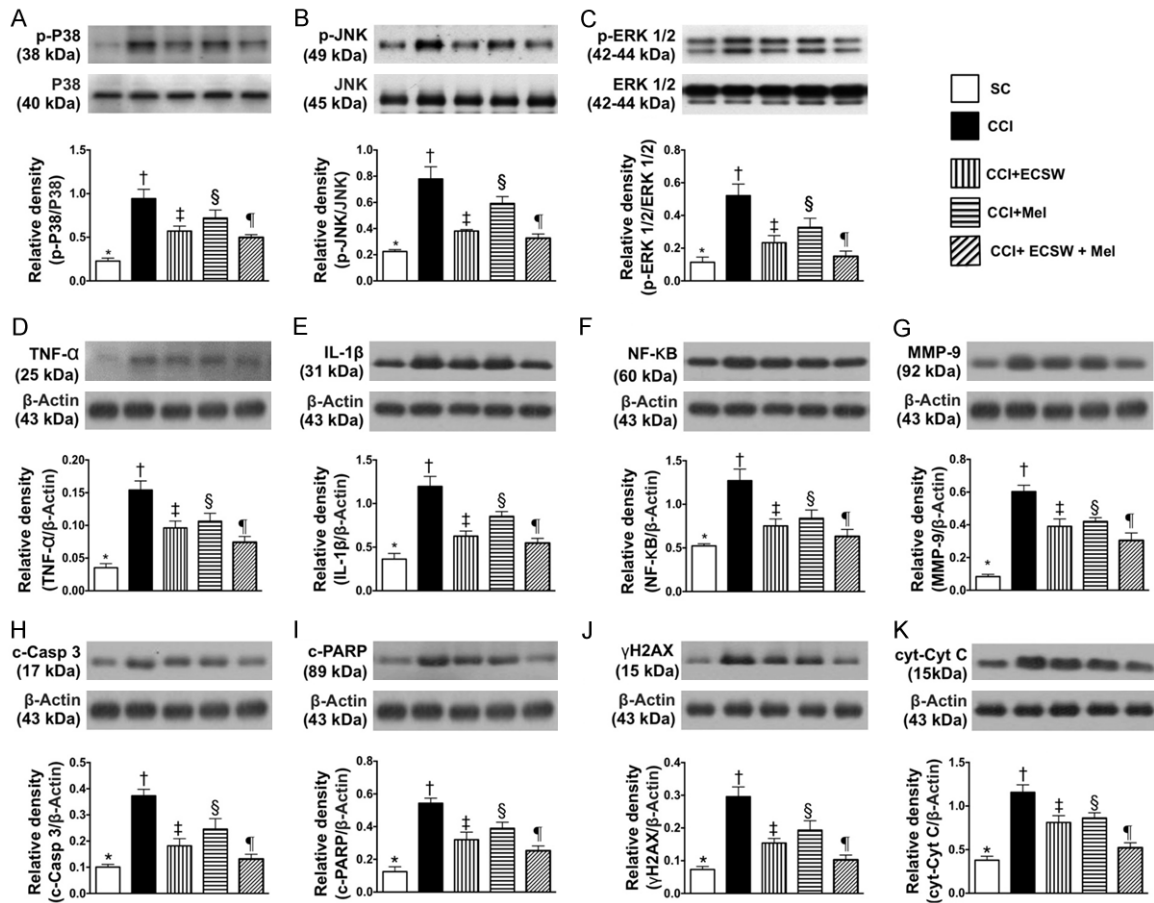


Figure 4. Protein expressions of signaling transduction molecules in L4-5 dorsal root ganglions (DRGs) and inflammatory, apoptotic, mitochondrial-damaged and DNA damaged biomarkers inspinal dorsal horn (SDH) by day 8 after CCI procedure. A. Protein expression of phosphorylated (p)-p38 in L4-5 DRGs, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. B. Protein expression of p-JNK in L4-5 DRGs, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. C. Protein expression of p-ERK1/2 in L4-5 DRGs, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. D. Protein expression of tumor necrosis factor (TNF)- α in SDH, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. E. Protein expression of interleukin (IL)-1 β in SDH, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. F. Protein expression of nuclear factor (NF)- κ B in SDH, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. G. Protein expression of matrix metalloproteinase (MMP)-9 in SDH, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. H. Protein expression of cleaved caspase 3 (c-Casp 3) in SDH, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. I. Protein expression of cleaved poly (ADP-ribose) polymerase (c-PARP) in SDH, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. J. Protein expression of γ -H2AX in SDH, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. K. Protein expression of cytosolic cytochrome C (cyt-Cyt C) in SDH, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test ($n = 8$ for each group). Symbols (*, †, ‡, §, ¶) indicate significance at the 0.05 level. SC = sham control; CCI = chronic constriction injury; ECSW = extracorporeal shock wave; Mel = melatonin.

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est in group 1, significantly higher in groups 3 and 4 than in group 5, and significantly higher in group 4 than in group 3.

Protein expressions of signaling transduction molecules in L4-5 DRGs and inflammatory, apoptotic, mitochondrial-damaged and DNA damaged biomarkers in spinal dorsal horn (SDH) by day 8 after CCI (Figure 4)

The protein expression of p-p38, p-JNK, p-ERK1/2 in L4-5 DRGs, three indicators of extracellular signal-regulated kinases (i.e., MAPK family) for response to stress stimulations, were lowest in group 1, highest in group 2, significantly higher in groups 3 and 4 than in group 5, and significantly higher in group 4 than in group 3.

The protein expressions of TNF- α and IL-1 β in SDH, two inflammatory biomarkers, were highest in group 2, lowest in group 1, significantly higher in groups 3 and 4 than in group 5, and significantly higher in group 4 than in group 3. Additionally, protein expressions of NF- κ B and MMP-9 in SDH, another two indicators of inflammation, showed a similar pattern to TNF- α among the five groups.

The protein expression of cleaved caspase 3 and cleaved PARP in SDH, two indicators of apoptosis, were highest in group 2, lowest in group 1, significantly higher in groups 3 and 4 than in group 5, and significantly higher in group 4 than in group 3. Additionally, the protein expressions of γ -H2AX in SDH, an indicator of DNA damage, exhibited an identical pattern to apoptosis among the five groups. Furthermore, protein expression of cytosolic cytochrome C in SDH, an indicator of mitochondrial-damage, displayed a pattern similar to γ -H2AX among the five groups.

Protein expressions of oxidative stress biomarkers and signaling transduction molecules, microglia and astrocyte activity in SDH by day 8 after CCI (Figure 5)

The protein expressions of NOX-1, NOX-2, NOX-4 and oxidized protein, four indicators of oxidative stress, were highest in group 2, lowest in group 1, significantly higher in groups 3 and 4 than in group 5, and significantly higher in group 4 than in group 3.

The protein expressions of p-p38, p-JNK, p-ERK1/2, three indicators of extracellular sig-

nal-regulated kinases, were lowest in group 1, highest in group 2, significantly higher in groups 3 and 4 than in group 5, and significantly higher in group 4 than in group 3.

The neuroinflammatory protein expressions of ox42, an indicator of microglial activation, and GFAP, an indicator of astrocyte activation, displayed an identical pattern to extracellular signal-regulated kinases among the five groups.

IF microscopy for co-localization of p-P38 and peripherin in DRG neurons (Figure 6)

To elucidate the presence of a peripheral nerve injury, the expression of p38 MAPK activation (i.e., phosphorylated p38) was measured. IF microscopy identified that p-P38 expression in peripherin, an indicator of small unmyelinated C-fiber and thinly myelinated A- δ fiber of DRG neurons that transmit signals of thermal and noxious stimuli, were lowest in group 1, highest in group 2, significantly higher in groups 3 and 4 than in group 5, and significantly higher in group 4 than in group 3.

IF microscopy for co-localization of p-P38 and NF200 in L4-5 DRG neurons (Figure 7)

IF microscopy identified that p-P38 expression in NF200, an indicator of large myelinated A- β fiber of DRG neurons that transmit information of non-noxious mechanical stimuli as well as abnormal mechanical allodynia in the pathologic neuropathic pain, was lowest in group 1, highest in group 2, significantly higher in groups 3 and 4 than in group 5, and significantly higher in group 4 than in group 3.

Discussion

This study investigated the impact of ECSW-Mel combination therapy on NP in rat and yielded several striking implications. First, MPWT and TPWL were increased in CCI animals compared to SC animals, suggesting that our experimental model was successfully created for the purpose of this study. Second, both MPWT and TPWL were significantly suppressed in CCI + ECSW-Mel animals compared to CCI only animals, and no ECSW-Mel-related complication was noted, highlighting both the safety and effectiveness of this combination treatment. Third, inflammation, oxidative stress, and MAPK family signaling pathway were found to be involved in NP and were markedly suppressed by ECSW-Mel therapy.

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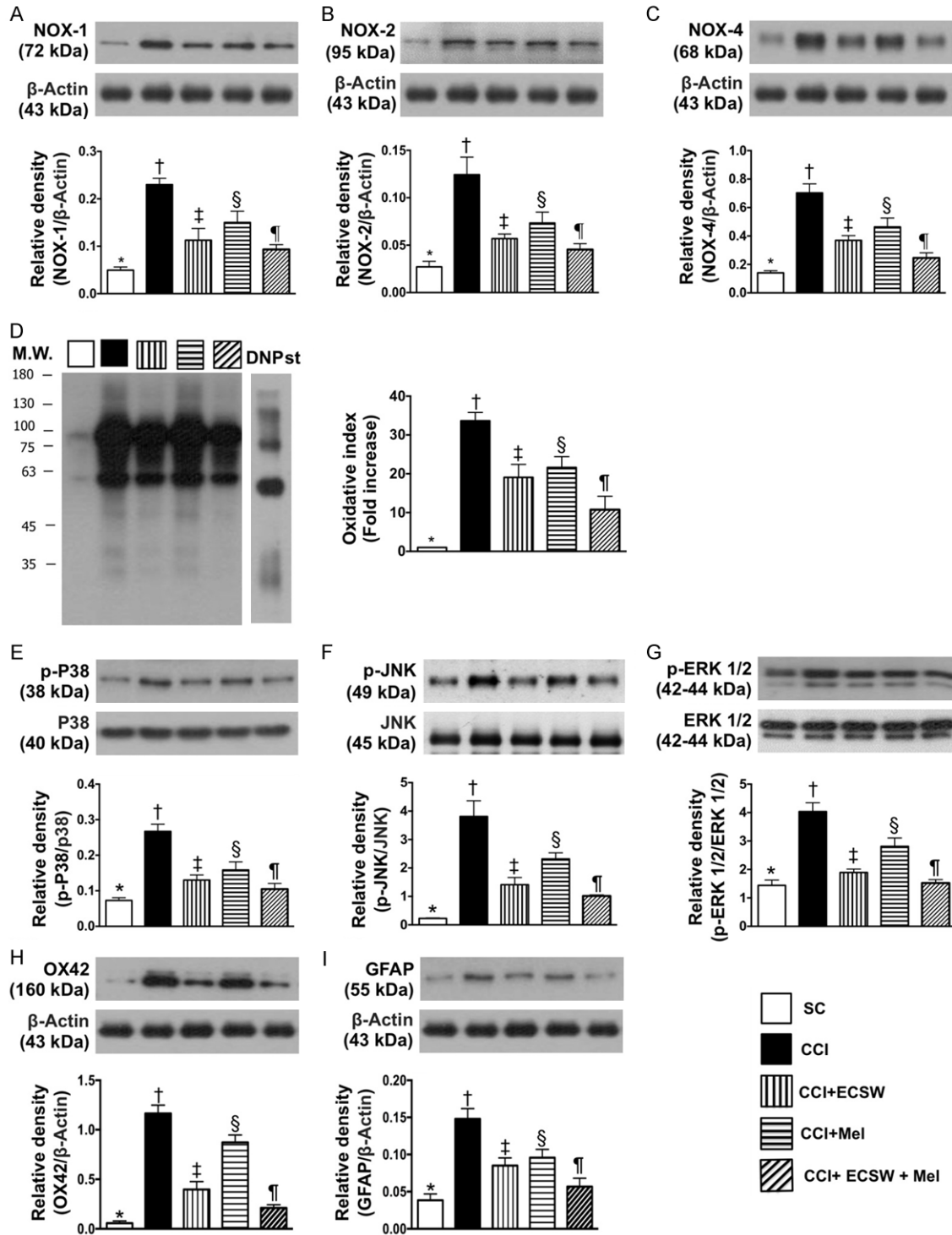


Figure 5. Protein expressions of oxidative stress biomarkers and signaling transduction molecules, microglia and astrocyte activity in spinal dorsal horn (SDH) by day 8 after CCI procedure. A. Protein expression of NOX-1, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. B. Protein expression of NOX-2, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. C. Protein expression of NOX-4, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. D. Oxidized protein expression, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. (Note: left and right lanes shown on the upper panel represent protein molecular weight marker and control oxidized molecular protein standard, respectively). M.W = molecular weight; DNP = 1-3 dinitrophenylhydrazone. E. Protein expression of phosphorylated (p)-p38, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. F. Protein expression of p-JNK, * denotes statistical significance vs. other groups

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with different symbols (†, ‡, §, ¶), $p < 0.0001$. G. Protein expression of p-ERK1/2, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. H. Protein expression of ox42, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. I. Protein expression of glial fibrillary acidic protein (GFAP), * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test ($n = 8$ for each group). Symbols (*, †, ‡, §, ¶) indicate significance at the 0.05 level. SC = sham control; CCI = chronic constriction injury; ECSW = extracorporeal shock wave; Mel = melatonin.

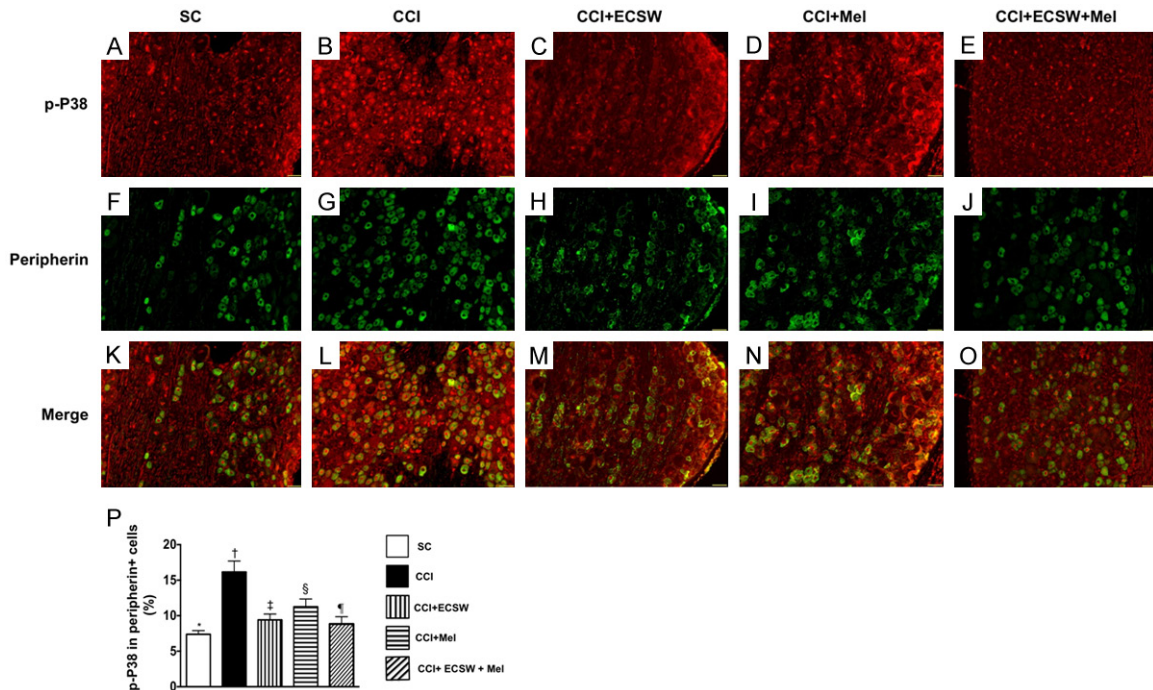


Figure 6. Immunofluorescent (IF) microscopic finding of colocalization of p-p38 and peripherin indorsal root ganglion (DRG) neurons. A-E. Illustrating the IF microscopic finding (200x) positively stained p-p38 in DRG neurons (red color spots). F-J. Illustrating the IF microscopic finding (200x) of merged positively-stained p-p38 and peripherin (green-red colocalization). K-O. Illustrating the IF microscopic finding (200x) of merged positively-stained p-p38 and peripherin (green-red colocalization). P. Analytical results of number of p-P38+/peripherin+ cells, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test ($n = 8$ for each group). Symbols (*, †, ‡, §, ¶) indicate significance at the 0.05 level. SC = sham control; CCI = chronic constriction injury; ECSW = extracorporeal shock wave; Mel = melatonin.

Clinical studies have previously shown that pain derived from the musculoskeletal system (i.e. tendonitis, plantar fasciitis, shoulder pain) were significantly inhibited by ECSW therapy [14-16]. Additionally, experimental studies have shown that Mel treatment inhibited NP [20-23]. The most important finding in the present study was that, as compared with CCI animals, the MPWT and TPWL, two standard tests for identifying the degree of NP, were found to be significantly attenuated in CCI animals treated with ECSW or Mel, and further significantly attenuated in CCI animals after receiving combination ECSW-Mel treatment. Accordingly, our findings extended those of previous studies [25, 26, 33, 35, 40, 41].

The underlying mechanisms involved in NP have been keenly investigated [3, 7, 8, 42-44] and are known to be complicated, involving persistent inflammation, generation of oxidative stress and elicitation of MAPK family signaling pathways [7-9, 12, 42, 45-51]. A principal finding in the present was that, as compared with SC group, the inflammatory reaction and generation of oxidative stress were markedly higher in the CCI group. Additionally, the MAPK family signaling pathways (i.e., p-p38, p-JNK, p-ERK1/2) were markedly upregulated in CCI animals than in SC animals. Therefore, our findings corroborated those of previous studies [45, 48, 51-53]. Importantly, the present study found that either ECSW or Mel treatment could

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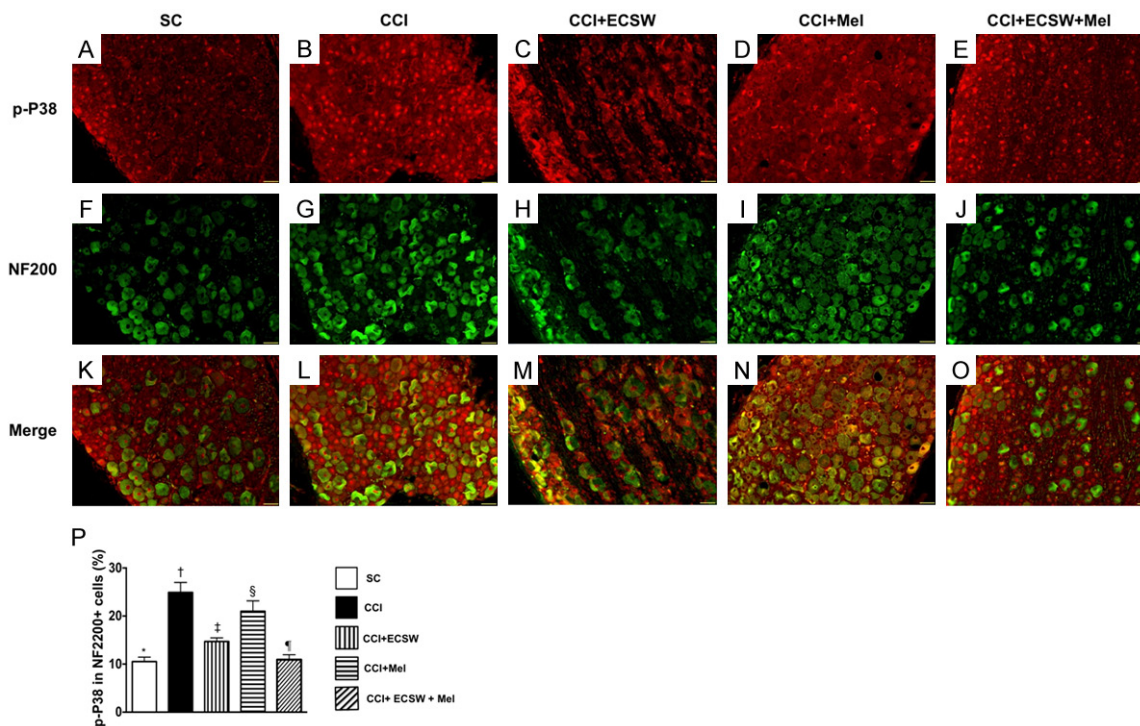


Figure 7. Immunofluorescent (IF) microscopic finding of colocalization of p-P38 and NF200 in dorsal root ganglion (DRG) neurons. A-E. Illustrating the IF microscopic finding (200x) positively stained p-P38 in DRG neurons (red color spots). F-J. Illustrating the IF microscopic finding (200x) positively stained NF200 cells (green color). K-O. Illustrating the IF microscopic finding (200x) of merged positively-stained p-P38 and NF200 (green-red colocalization). P. Analytical results of number of p-P38+/NF200+ cells, * denotes statistical significance vs. other groups with different symbols (†, ‡, §, ¶), $p < 0.0001$. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test ($n = 8$ for each group). Symbols (*, †, ‡, §, ¶) indicate significance at the 0.05 level. SC = sham control; CCI = chronic constriction injury; ECSW = extracorporeal shock wave; Mel = melatonin.

significantly suppress the inflammatory reaction and generation of oxidative stress in SDH and DRG neurons of CCI animals. Of particular importance was that combined ECSW-Mel was superior to either therapy alone at suppressing the expressions of inflammation and oxidative stress. In this way, our findings support the reports of synergism from combined therapy in previous studies [25, 26, 33, 35, 40, 41], and also explain why MPWT and TPWL were substantially ameliorated in CCI animals after receiving ECSW-Mel treatment.

An association between inflammation/oxidative stress and DNA/mitochondrial damage has been well recognized [7-10, 12, 17, 47]. Intriguingly, a link between inflammation/oxidative stress and DNA/mitochondrial damage as well as apoptotic biomarkers in the present study were notably higher in CCI animals than in SC animals. However, these biomarkers were downregulated by ECSW or Mel treatment and

further downregulated by combination ECSW-Mel. Our findings, in addition to reinforcing the previous reports [25, 26, 33, 35, 40, 41], implicated that ECSW-Mel therapy effectively attenuated the DNA/mitochondrial damage through the suppressing the role of inflammation/oxidative stress reaction. Our previous study demonstrated that ECSW therapy protected the sciatic nerve against diabetic-induced neuropathy [17] by inhibiting the inflammatory reaction and oxidative stress and DNA/mitochondrial damage. Accordingly, the findings of our previous study [17] were consistent with those from this current study.

Nav.1.3, Nav.1.8 and Nav.1.9 are three indicators of voltage-gated sodium channels of sciatic nerve/DRG neurons for ectopic discharges or activities in response to mechanical and thermal stimulations [54, 55]. Their protein expressions in sciatic nerve/DRG neurons were markedly increased in CCI animals compared to SC

animals. Additionally, the co-existing p-P38-peripherin+ and p-P38-NF200+ cells in DRG neurons were substantially higher in the CCI groups than in the SC group. Our findings are thus comparable with those from previous studies [45, 46, 48, 53] and could, at least in part, explain why MPWT and TPWL were significantly higher in CCI animals than in SC animals. However, these two parameters were notably suppressed by ECSW or Mel treatment and further suppressed by ECSW-Mel combined treatment, highlighting that the regulation of voltage-gated sodium channels and the expressions of p-P38+/peripherin+ and p-P38+/NF200+ cells were crucial for stifling NP.

Study limitation

This study has limitations. First, although outcomes observed in the present study were promising, the study period was only eight days in duration. Therefore, long-term outcomes from the present study remain uncertain and invite further study. Second, although extensive work was done in the present study, the exact underlying mechanisms of ECSW-Mel therapy for relieving NP remain unclear.

In conclusion, the present study demonstrated that ECSW-Mel combination therapy effectively ameliorated NP in rat. These findings raise the need for a prospective clinical trial to answer whether this therapeutic option is also effective for patients with NP that is refractory to conventional therapy.

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Disclosure of conflict of interest

None.

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