

Original Research

Spinal Cord Stimulation Alleviates Pain Hypersensitivity by Attenuating Neuroinflammation in a Model of Painful Diabetic Neuropathy

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Abstract

Introduction: Painful diabetic neuropathy (PDN) is an intractable chronic pain condition affecting a growing number of adults in China. Spinal cord stimulation (SCS) has been employed in the treatment of PDN for several decades. However, the efficacy and underlying mechanisms of SCS are still inconclusive. **Methods:** In this study, we adopted an implantable pulse generator to deliver electrical stimulation (50 Hz, 200 μ s pulse width, 12 hours/day in 5 weeks) via a quadripolar electrode in the lumbar epidural space to treat pain hypersensitivity in the rat model of PDN. Electronic von Frey and Hargreaves tests were used to measure the responses to mechanical and heat stimuli, respectively. Quantitative PCR, western blotting, and enzyme-linked immunosorbent assay (ELISA) were adopted to explore the changes in neuroinflammation after SCS. **Results:** SCS alleviated mechanical allodynia and heat hyperalgesia over a period of 3 weeks in diabetic rats. SCS completely suppressed neuropathy-induced Tlr4 and NF κ B p65 elevation, resulting in the reduction of pain-promoting Il1 β , Il6, and Tnf α proteins in the spinal cord dorsal horn. **Conclusions:** SCS may alleviate diabetic neuropathy-induced pain hypersensitivity via attenuating neuroinflammation in the spinal cord dorsal horn.

Keywords: diabetic neuropathies; spinal cord stimulation; chronic pain

1. Introduction

The prevalence of diabetes is increasing in China. It was estimated that total diabetes and newly diagnosed diabetes account for 12.8% (12.0–13.6%) and 6.8% (6.1–7.0%) of adults in China [1]. Meanwhile, 50% of these patients may develop peripheral neuropathy in their lifetime [2] and up to 50% of patients with diabetic neuropathy may suffer from neuropathic pain syndrome [3]. Hence the painful diabetic neuropathy (PDN) gains growing attention [4]. Unfortunately, the pharmacological treatment for PDN is still not satisfactory in clinical practice.

Diabetic neuropathy is characterized by the loss of small fibers in the skin and is generally regarded as peripheral neuropathy. Pathological alterations in the dorsal root ganglion (DRG) are one of the most discernible etiologies of painful neuropathy. For instance, it was reported that 1/3 of c-fibers (nociceptors) exerted hyperexcitability in response to mechanical stimuli, resulting in an up to a 3-fold increase in firing frequency [5]. Cxcl12 appeared to cause the hyper-responsiveness of c-fibers by activating Cxcr4 [6]. Recently, the investigation of the plasma of patients with diabetes revealed that patients with PDN had significantly higher level of methylglyoxal than patients without pain. Moreover, methylglyoxal dramatically increased

the excitability and firing rate of nociceptors via modulating Nav1.8 and Nav1.7 [7]. These studies suggested that the alterations in peripheral inflammation or metabolomics might evoke pain hypersensitivity via targeting at DRG. In addition, the pathological changes in the spinal cord are also discerned, given that pain hypersensitivity is an evident clinical manifestation. For instance, long-term hyperglycaemia was reported to cause microvascular complications in the spinal cord and associated with pain-like responses in the preclinical models of PDN [8]. Meanwhile, the increased number of synapses [9], oxidative stress related damage [10], infiltration of blood-borne macrophages [11] and reactivation of microglia [12] were identified as contributing factors of PDN. In this connection, spinal cord is recognized as a potential target for PDN intervention.

Since few decades ago, electrical spinal cord stimulation (SCS) has been applied as an approach for PDN therapy [13]. In this pilot study, 10 patients with treatment-refractory PDN received SCS treatment and achieved significant pain relief for up to 6 months. Numerous clinical studies further presented a promising efficacy of SCS on PDN [14,15]. A long-term follow-up study had reported that SCS led to 50% pain reduction in 55% participants for as long as 5 years [16]. However, according to the recom-



mendations of the international association for the study of pain Neuropathic Pain Special Interest Group (NeuPSIG), the effects of SCS on PDN is still inconclusive due to relative low quality of clinical trials [17]. The clinical guideline for the SCS treatment of PDN, such as the recommended electrical frequency, pulse width, duration of stimulation, electrode configuration, was not developed. Further studies that aim to modify the stimulation protocols could improve the therapeutic efficacies and reduce the safety concerns in the clinical practice. In this regard, preclinical studies adopting biochemistry based methodologies to objectively evaluate the changes in pain associated molecules serve as valuable approaches to fine tune SCS protocols.

In this study, the efficacy of a SCS protocol in the pain-like hypersensitivity was investigated on the rat models of PDN. The changes in pain-associated molecules, i.e., inflammatory cytokines and their upstream modulators, were also assessed after SCS.

2. Methods and Materials

2.1 Animals

Male Sprague Dawley (SD) rats, weighting 225 g was used in all presented experiments. Rats were kept in the animal room at a temperature of 24 °C, relative humidity of 60% and 12:12-hour light: dark cycle. All the procedures were approved by the animal ethical committee of Jingzhou Hospital Affiliated to Yangtze University (approval number: JZYY-2020142).

2.2 Rat Model of Painful Diabetic Neuropathy

The diabetic neuropathy was induced via intraperitoneal (i.p.) injection of streptozotocin (STZ) as described by previous report [18]. STZ was obtained from TOCRIS (No. 1621, Bio-Techne China, Shanghai, China) and dissolved with sodium citrate buffer (10 mM, pH5.5) to make a 50 mg/mL working solution. For model development, rats were fasted for 6 hours and then injected with a single dose of STZ (45 mg/kg) using a 1 mL syringe. For sham group, rats were administrated with sodium citrate buffer. After injection, all rats were given 10% sucrose water in a period of 48 hours to prevent hypoglycaemics.

2.3 Measurement of Blood Glucose Levels

AccuChek Performa (Roche Diagnostics, Mannheim, Germany) was adopted to measure non-fasting blood glucose of rats. The blood was taken by puncturing the tip of tail with a hypodermic needle and loaded onto the test strip for measurement. The test was conducted before STZ injection and every week from day 21 to day 49 after injection. The body weight was then recorded after blood test.

2.4 Electronic Von Frey Test

The allodynia-like response of rats were measured with an electronic von Frey aesthesiometer (No. 76-0976, Harvard Apparatus, Holliston, MA, USA). The rats were

placed in the light chamber on the wire mesh stand. Rats were allowed to acclimate for 15 minutes before test. For measurement, the researcher stabbed the central plantar of rats with the supplied tip and lifted until a pain-like response (i.e., withdrawal, and linking) was observed. The withdrawal threshold of mechanical stimulation was shown in the von Frey meter and recorded. For each time point, all the rats were tested for 5 times in a 5 minutes intervals. The researchers were blinded to the grouping information.

2.5 Hargreaves Heat Hyperalgesia Test

The IITC plantar analgesia meter (390G, IITC life science, Woodland Hills, CA, USA) was applied to measure the changes in heat-evoked response before and after STZ injection. Rats were place in the animal enclosure and allowed to acclimate for 15 minutes before test. A beam of light, which may deliver noxious heat, was aimed at the central plantar of rats until a pain-like response was observed (withdrawal or linking), and the paw withdrawal latency was recorded. For each time point, all the rats were tested for 5 times in a 5 minutes intervals. The researchers were blinded to the grouping information.

2.6 Electrical Spinal Cord Stimulation (SCS)

The quadripolar SCS electrode and Interstim-II Implantable Pulse Generator (IPG) was obtained from Medtronic (Model 8840, Minneapolis, Minnesota, MN, USA) and implanted on rats from all groups, i.e., control group (without STZ injection), the STZ+sham therapy group (n = 6) and STZ+SCS therapy group (n = 6), at week 3 after i.p. injection. Rats were deeply anaesthetized with isoflurane. A partial laminectomy was performed at the vertebrate level of L1. The electrode was then inserted towards T13 vertebrate level to cover the lumbar spinal cord. The lead of electrode was fixed to the muscles with sutures to secure its position. A IPG was then placed subcutaneously near L1 vertebrate and connected to the lead. After implantation, the muscles and the skins were closed separately. To perform SCS, N'Vision clinician programmer (model 8840, Medtronic, Minneapolis, MN, USA) was adopted to control the electrical delivery by the IPG. First of all, motor threshold was determined by gradually increasing the current amplitude (4 Hz at 200 us pulse width) from 0, until reflection was observed in the hind limb or trunk. For SCS, current amplitude at 80% motor threshold, i.e., 50 Hz at 200 us pulse width was continuously delivered for 12 hours daily, from week 4 after STZ injection, to the end of experiments. For control group and sham therapy group, no currents were delivered.

2.7 Quantitative PCR (qPCR)

The relative expression level of Tlr4 and NF- κ B p65 gene were determined using the qPCR method. Total RNA from lumbar spinal cord dorsal horn tissues were isolated with Trizol reagent (No. 15596026, Thermo Fisher Sci-

entific, San Jose, CA, USA). Dissected tissues were lysed in 1 mL Trizol reagent, following by protein and genomic DNA removal with 200 μ L chloroform and total RNA precipitation with 500 μ L isopropanol. Complementary DNA (cDNA) was synthesized with 1 μ g of total RNA from each rat using the PrimeScript RT reagent kit (No. RR037A, Takara, Dalian, China) according to manufacturer's instruction. For qPCR, cDNA was diluted for 30 folds with perfect diluent (provided by the kit) and then used for amplification with QuantiTect SYBR Green RT-PCR kit (No. 204156, Qiagen Sciences, Germantown, MD, USA) in an ABI 7900HT system (Thermo Fisher Scientific, San Jose, CA, USA). The relative gene expression levels were normalized to ACTB expression using the $2^{-\Delta\Delta C_t}$ method.

2.8 Western Blotting

The protein level of Tlr4, phosphorylated and NF- κ B p65 was measured by western blotting. Total protein was extracted from lumbar spinal cord dorsal horn tissues using ReadyPrepTM protein extraction kit (No. 1632086, Bio-Rad, Hercules, CA, USA). Protein concentration was detected using Enhanced BCA protein assay kit (No. 23225, Beyotime, Shanghai, China). Protein samples (10 μ g/lane) was loaded and separated on SDS-PAGE, then transferred onto 0.22 μ m PVDF membranes (3010040001, Roche, Nottingham, UK). The membrane was blocked with 5% skim milk in TBST (20 mM Tris, pH8.0, 137 mM NaCl and 0.1% Tween-20) and incubated with primary antibodies (1:1000 dilution) against Tlr4 (#14358S, Cell Signaling, Danvers, MA, USA), phosphor-NF- κ B p65 (S536, #3033, Cell Signaling, Danvers, MA, USA), NF- κ B p65 (#8242, Cell Signaling, Danvers, MA, USA) and β -actin (#3700, Cell Signaling, Danvers, MA, USA) for overnight at 4 °C, following by incubation of corresponding secondary antibodies (1:3000 dilution, Cell Signaling, Danvers, MA, USA) for 1 hour at room temperature. All the antibodies were purchased from Cell signaling technology (Danvers, MA, USA). Finally, the target bands were visualized using SuperSignal West Dura (ThermoFisher Scientific, San Jose, CA, USA) and quantified with Image J (NIH, Baltimore, Maryland, USA).

2.9 Enzyme-Linked Immunosorbent Assay (ELISA)

The protein level of proinflammatory cytokines were measured by ELISA. All the ELISA kits, including rat Il1 β (SRLB00, Bio-techne China, Shanghai, China), Il6 (SR6000B, Bio-techne China, Shanghai, China) and Tnf α (SRTA00, Bio-techne China, Shanghai, China) quantikine ELSIA kits were purchased from R&D systems (Bio-techne China, Shanghai, China). Spinal cord dorsal horn tissues were collected and lysed using RIPA buffer (ab156034, Abcam, Cambridge, MA, USA) supplemented with protease inhibitor cocktails (ab65621, Abcam, Cambridge, MA, USA). The protein concentration was determined as described above. All the samples were then adjusted to

the same concentration with RIAP buffer and loaded to the wells of ELISA plates for 1.5 hours at room temperature. After three washes, Il1 β , Il6 or Tnf α antiserum was added and incubated for 30 min, following with another 3 x washes and antibody conjugate incubation for 30 min. Finally, the substrate solution and stop solution were applied to determine the content of cytokines at 570 nm.

2.10 Statistics

For analysis of three groups across time, two-way analysis of variance (ANOVA) followed by multiple comparisons were conducted. For analysis of four groups at a single time point, one-way ANOVA with multiple comparisons were employed. A *p*-value less than 0.05 was considered statistical significance.

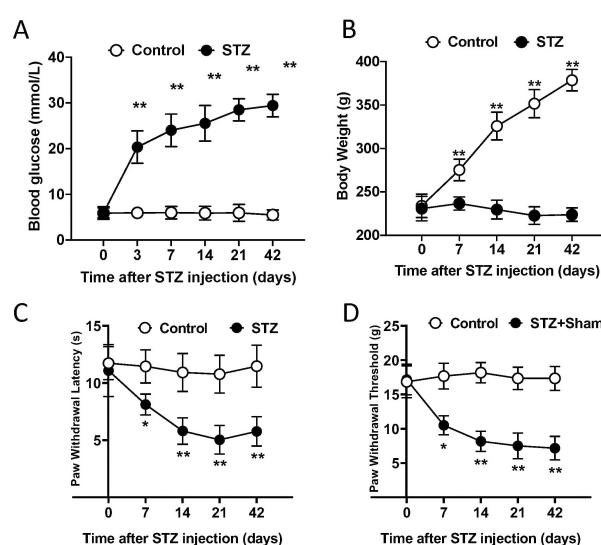


Fig. 1. Single injection of STZ developed painful diabetic neuropathy. (A) Blood glucose test demonstrated STZ injected rats had significantly higher glucose levels than control rats from day 21 to day 49 after injection. (B) STZ injection interfered with gain of body weight. (C) STZ treatment induced heat hyperalgesia on rats from 4 weeks after injection. (D) STZ treatment induced mechanical allodynia on rats from 4 weeks after injection. A and B, Student's *t* test. C and D, Two-way ANOVA with post hoc test. *, *p* < 0.05; **, *p* < 0.01. *n* = 6 rats/group. The error bars represent standard deviation.

3. Results

3.1 Single Injection of STZ Developed Painful Diabetic Neuropathy

Rats (*n* = 12) were assessed for the baseline of paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) by electronic von Frey test and Hargreaves heat hyperalgesia test, respectively. Rats were then randomly assigned to control (vehicle treated) or STZ treated groups according to the baseline. As expected, the blood glucose

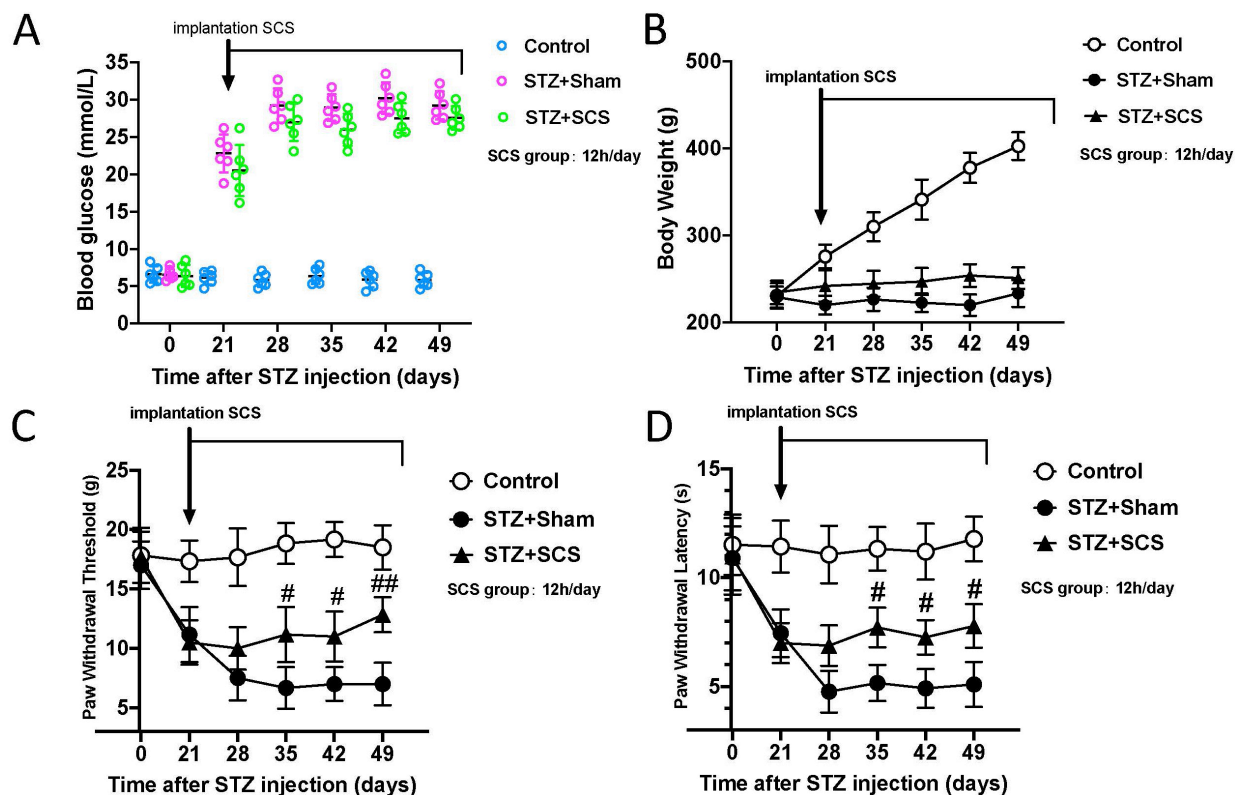


Fig. 2. SCS alleviated pain hypersensitivity without affecting hyperglycemia. (A) Blood glucose levels were not affected by SCS. (B) SCS did not restore body weight gain. (C–D) SCS alleviated mechanical allodynia and heat hyperalgesia after 2 weeks and lasted for at least 3 weeks. Two-way ANOVA with post hoc test. #, $p < 0.05$; ##, $p < 0.01$. $n = 6$ rats/group. The error bars represent standard deviation.

levels were dramatically elevated in STZ group, compared with the control group. The hyperglycemia was observed at week 3 after STZ injection and lasted for at least for 5 weeks (Fig. 1A). Meanwhile, the body weight was gradually gained in the control group, whereas it was not in the STZ group (Fig. 1B). These results indicated the development of type I diabetes in rats after STZ injection. Furthermore, STZ rats have significantly lower PWT and PWL than control rats, from week 4 to week 7 after model development (Fig. 1C,D). Taken together, we successfully developed the rat model of painful diabetic neuropathy.

3.2 SCS Alleviated Pain Hypersensitivity without Affecting Hyperglycemia

We then explored if SCS could improve diabetic neuropathy-induced mechanical allodynia and heat hyperalgesia. Rats ($n = 18$) were assessed for baseline of pain-like response and randomly assigned to three groups, i.e., control group, STZ+sham therapy group and STZ+SCS therapy group. The electrode and IPG implantation were conducted on rats from all groups at day 21 after model development. Then SCS (12 hours daily, 50 Hz, 200 μ s pulse width) or sham therapy began from day 21 to the end of experiments. As demonstrated in the Fig. 2A,B, SCS did not improve hyperglycemia and increase body weight among STZ treated

rats. There was no significant difference in pain hypersensitivity between sham and SCS groups at post-injection day 21 and day 28. However, the therapeutic efficacy of SCS was evident at day 35, day 42 and day 49, as indicated by a significant increase of both PWL and PWT in SCS group, compared with sham group (Fig. 2C,D). These results suggested that SCS was an efficient approach for relieving diabetic neuropathy-induced pain.

3.3 SCS Suppressed *Tlr4/NF- κ B p65* Pathway in Spinal Cord Dorsal Horn

Neuroinflammation has been closely associated to the development of central sensitization, which contributes to allodynia and hyperalgesia in various model of neuropathic pain [18,19]. Therefore, the effects of SCS on neuroinflammation were investigated in this study. qPCR was performed to assess the changes of *Tlr4* and *NF- κ B p65* genes. After STZ injection, the mRNA levels of both *Tlr4* and *NF- κ B p65* were significantly increased in spinal cord dorsal horn, compared with control group (Fig. 3A,B). Notably, SCS for 5 weeks completely suppressed the up-regulation of *Tlr4* and *NF- κ B p65* (Fig. 3A,B). There was no significant difference between control and STZ+SCS group, indicative of suppression of *Tlr4/NF- κ B p65* pathway. This finding was further confirmed by the western blotting assay,

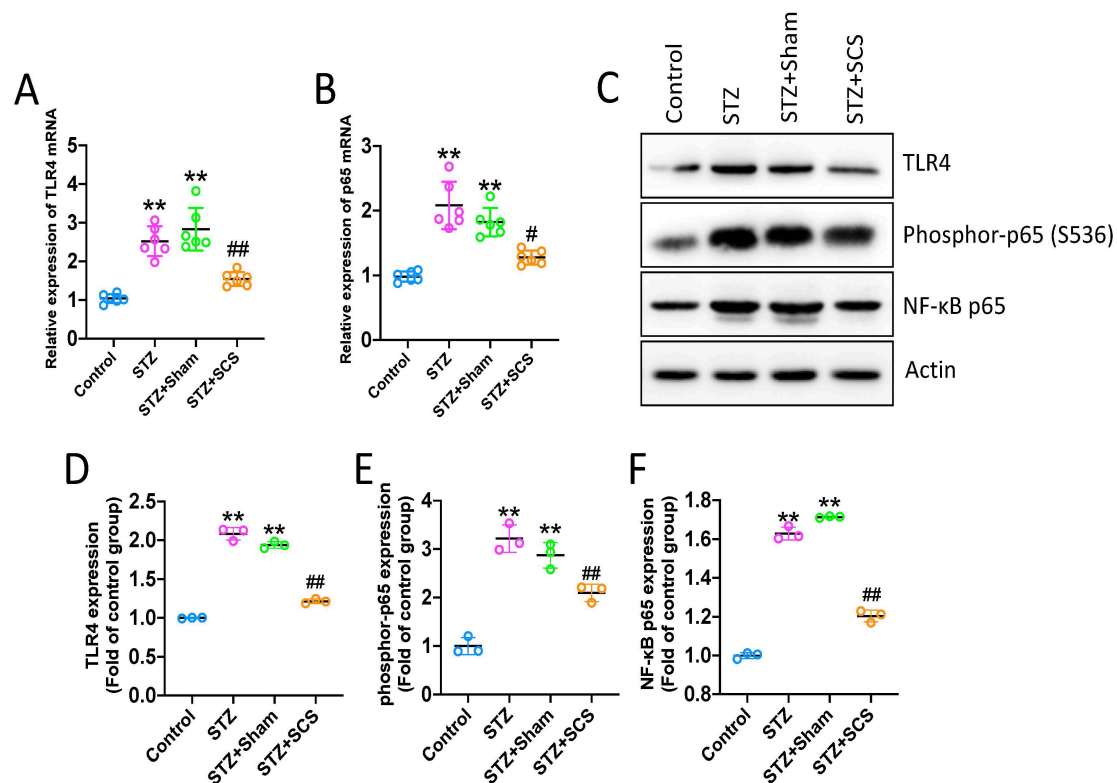


Fig. 3. SCS suppressed Tlr4/NF- κ B p65 pathway in spinal cord dorsal horn. (A–B) Compared with control rats, diabetic rats had significantly higher mRNA of Tlr4 and NF- κ B p65. SCS, but not sham stimulation suppressed STZ induced gene elevation. (C–F) Western blotting confirmed the alterations of Tlr4, phosphor-p65 and NF- κ B p65 among groups. One-way ANOVA with post hoc test. **, $p < 0.01$, compared with control group; #, $p < 0.05$, compared with STZ+sham group. $n = 6$ rats/group. The error bars represent standard deviation.

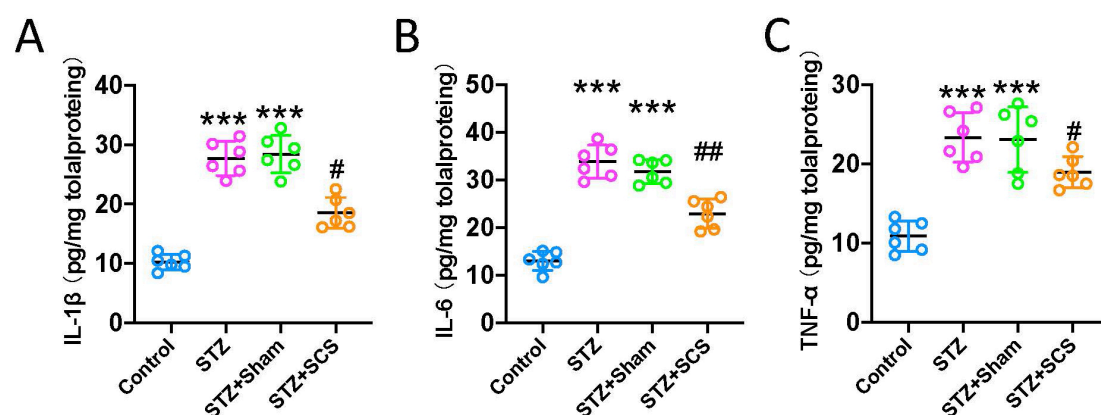


Fig. 4. SCS reduced pro-inflammatory cytokines in spinal cord dorsal horn. (A–C) ELISA demonstrated that diabetic rats had significantly higher levels of IL-1 β , IL-6 and Tnf α than control rats. SCS, but not sham stimulation suppressed STZ induced up-regulation of these three cytokines. One-way ANOVA with post hoc test. ***, $p < 0.001$, compared with control group; #, $p < 0.05$, ##, $p < 0.01$, compared with STZ+sham group. $n = 6$ rats/group. The error bars represent standard deviation.

which demonstrated that STZ and STZ+sham therapy group had significantly higher level of Tlr4, phosphor-NF- κ B p65 and NF- κ B p65 proteins than control group, whereas SCS reversed the elevation of Tlr4/NF- κ B p65 (Fig. 3C–F).

3.4 SCS Reduced Pro-Inflammatory Cytokines in Spinal Cord Dorsal Horn

Next, the pro-inflammatory targets of Tlr4/NF- κ B p65 pathway was assessed. In line with the alterations in Tlr4/NF- κ B p65 pathway, ELISA showed that STZ

also caused significant increase of $Il1\beta$, $Il6$ and $Tnfa$ (Fig. 4A–C), which play critical roles in central sensitization. SCS for 5 weeks greatly reduced the elevation of these pro-inflammatory cytokines, as compared with STZ or STZ+sham therapy group (Fig. 4A–C). This result consolidated the suppression of neuroinflammation by SCS.

4. Discussion

In the present study, the therapeutic efficacy and potential mechanism of SCS in painful diabetic neuropathy was investigated. We found that daily SCS for 5 weeks successfully reduced pain hypersensitivity caused by diabetic neuropathy. The therapeutic action was accompanied by the decline of $Tlr4$ and $NF-\kappa B$ p65 mRNA and proteins, as well as their proinflammatory target genes, i.e., $Il1\beta$, $Il6$ and $Tnfa$ in spinal cord dorsal horn. We concluded that SCS could improve painful diabetic neuropathy by mitigating neuroinflammation.

Recently, the pain hypersensitivity associated pathological changes in spinal cord had gained more and more attentions, particularly the dysregulated inflammatory responses. Microglia are the glia sensing injury or dysregulated activation in central nervous system. In the model of STZ induced neuropathic pain, microglia are rapidly aroused and the re-activation status could last for as long as 6 months [20]. Re-activated microglia subsequently modulate neuroinflammation by secreting a series of cytokines, such as $Il1\beta$, $Il6$ and $Tnfa$, leading to the enhanced excitability of neurons and the increased strength of synapses in spinal cord dorsal horn [12,21]. Administration of microglial inhibitor minocycline led to the suppression of pro-inflammatory cytokines, and efficiently improve pain-like responses of diabetic animals [22,23]. Meanwhile, accumulating pharmacological studies further consolidated neuroinflammation inhibition as a potent treatment of painful diabetic neuropathy [24–26]. $Tlr4/NF-\kappa B$ signaling pathway is critical for the elevation of pro-inflammatory cytokines in various cell types under distinct pathological conditions, and had been considered an effective target for diseases treatment [27–30]. In this study, we found that STZ induced neuropathy was accompanied with increased $Tlr4$, phosphor- $NF-\kappa B$ p65, and total $NF-\kappa B$ p65 levels, indicative of increased inflammatory responses in spinal cord. It is well known that $NF-\kappa B$ p65 phosphorylation at S536 leads to the increased acetylation by CREB-binding protein (CBP) CBP/p300 proteins, following enhanced transactivation of cytokine genes, including $Il1\beta$, $Il6$, and $Tnfa$. Meanwhile, several phosphorylation sites, such as S468 and T254, are associated with $NF-\kappa B$ p65 stability. Our results indicated that post-translational modifications could have been occurred at multiple sites of $NF-\kappa B$ p65 and synergistically enhance $NF-\kappa B$ p65-mediated transcription by increasing its stability and transactivation. Indeed, ELISA confirmed the marked elevation of $Il1\beta$, $Il6$ and $Tnfa$. On the other hand, SCS for 5 weeks significantly reduced the

mRNA and proteins levels of $Tlr4$ and $NF-\kappa B$ p65, as their downstream pain-promoting cytokines. We therefore concluded that the pain alleviating effects of SCS is at least partially mediated through the suppression of $Tlr4/NF-\kappa B$ p65-dependent neuroinflammation in spinal cord dorsal horn.

The potential connection between neuroinflammation suppression and SCS might be useful for clinical trial design and further, for patient selection in the future clinical practice. By use of simultaneous positron emission tomography/magnetic resonance (PET/MR) imaging technique and the radiolabeled ligand of translocator protein (translocator protein, TSPO, a glia cell related inflammatory marker), spinal cord and nerve roots related neuroinflammation can be evaluated and associated with chronic pain conditions [31]. Clinical scientists are encouraged to use this technique and set up clinical trials testing if SCS analgesia is associated with the attenuated neuroinflammation in patients. Meanwhile, it is also worth investigating if patients with higher degree of neuroinflammation are more likely to be sensitive to SCS treatment. On this basis, we may recruit PDN patients having significant neuroinflammation to the priority group for SCS treatment in the clinical practice. On the other hand, after consolidating the relationship of SCS analgesia and neuroinflammation in humans, it may reinforce researchers' confidence to seek novel approaches to targeting reactivated glia cells or neuroinflammation for PDN treatment.

There are some limitations in the current reports. While SCS exerted considerable analgesic-like actions within the duration of electrical stimulation, we did not measure the post-treatment pain thresholds after 5 weeks. It is likely that intervention on neuroinflammation may prevent the buildup of long-term central sensitization [19,32]. Meanwhile, several independent studies had reported the efficacies of SCS to neuropathic pain by adopting differential frequencies [33–35]. In this study, only one protocol, i.e., 50 Hz was employed since a randomized controlled trial has reported that high-frequency 10 kHz SCS improves the quality of life in patients with PDN as well [36]. Therefore, further study focusing on the comparison of therapeutic efficacies between different protocols (for instance, 50 Hz vs 10 kHz) might provide pre-clinical evidence for the improved design of clinical trials. Finally, although neuroinflammation suppression has been delineated as potential mechanism of SCS, we did not pinpoint the source of cytokines (e.g., microglia and astrocytes). Previous studies suggested that microglia appeared to play critical roles only in the initiation of neuropathic pain, whereas astrocytes were activated and contributed to the maintenance of neuropathic pain [37,38]. A precise cell type localization therefore may advise the time window of SCS delivery and refine the design of clinical trials. We will explore these open questions in the future studies.

5. Conclusions

SCS may alleviate diabetic neuropathy-induced pain hypersensitivity via attenuating neuroinflammation in the spinal cord dorsal horn.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

WN, NW, YW—extraction and drafting of the manuscript; WN, JL, QX, NW—analysis of data, manuscript revision; NW, and YW—design and revision, statistical analysis. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All the procedures were approved by the animal ethical committee of Jingzhou Hospital Affiliated to Yangtze University (approval number: JZYY-2020142).

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Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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