The Application of Pulsed Radiofrequency to Dorsal Root Ganglion Ameliorates Neuropathic Pain by Reducing CCL2 Expression in the Early Stage After Sciatic Nerve Injury in Rats

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Research Article

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Abstract

Background: Neuropathic pain (NP) can be treated effectively using pulsed radiofrequency (PRF). NP development and maintenance involves the essential neurotransmitter chemokine c-c motif ligand 2 (CCL2). The present study aimed to determine whether PRF regulated CCL2 expression in sciatic nerve injury (SNI) model rats.

Methods: Sprague-Dawley rats were divided randomly into a sham group, an SNI group, and a PRF group. In the PRF group, L5 dorsal root ganglia received PRF treatment. After paw withdrawal mechanical threshold (PWMT) was examined, the expression levels of CCL2 and nuclear factor kappa B (NF-κB) in spinal dorsal horn were determined.

Results: The PWMT in PRF group increased significantly compared with that of the SNI group (P < 0.05). The CCL2 and NF-κB expression levels in the PRF group were significant lower than those in the SNI group (P < 0.05).

Conclusion: NP was effectively alleviated by PRF-mediated reductions in CCL2 expression via inhibition of NF-κB activation in the spinal cord of SNI model rats.

Background

Neuropathic pain (NP) refers to pain that is started by or induced by a dysfunction or lesion in the somatosensory system. The significant morbidity associated with NP represents a challenge to public health, especially for elderly patients. Although many studies have investigated NP development and maintenance mechanisms, the specific details are lacking. Thus, NP remains a clinically refractory pain that requires further research.

Recently, the method of pulsed radiofrequency (PRF) has been used to treat pain because of its low or absent induction of neurodestruction. PRF is delivered in a pulse of 20 milliseconds (ms) followed by a silent period of 480 ms to avoid radiofrequency heat lesions. PRF is typically believed to modulate pain pathology and clinical studies have proven its ability to treat several types of NP, such as postherpetic neuralgia, painful diabetic neuropathy, and lumbar surgery failure syndrome. In addition, PRF can reduce NP by regulating numerous cytokines, as demonstrated using animal studies. However, the mechanism underlying this observation remains unknown.

The chemokine family member chemokine C-C motif ligand 2 (CCL2, also termed monocyte chemoattractant protein 1) plays a key role in the chemotaxis of monocytes to sites of trauma, infection, toxin exposure, and ischemia. CCL2 shows low expression under normal conditions, but is upregulated rapidly in the early stage of nerve injury. The expression levels of CCL2, together with its receptor (CC chemokine family receptor 2, CCR2), increased in the dorsal horn of the spinal cord in an NP model rat. Interestingly, in non-inflamed rats, CCL2 intrathecal injection resulted in obvious nociceptive pain behavior.
Moreover, intrathecal preemptive treatment with a CCR2 antagonist markedly reduced nerve injury-induced pain behavior and inflammatory cytokine release. Thus, there is an implication that CCL2 and CCR2 have critical functions in NP development and maintenance after nerve injury. However, whether PRF reduces NP by modulating CCL2 expression is unknown. The present study aimed to assess the effects of direct PRF application on dorsal root ganglia (DRGs) in the early stage in sciatic nerve injury (SNI) model rats and to determine whether PRF modulates CCL2 expression in the spinal cord.

Materials And Methods

Experimental animals and groups

The animal care and use committee of China Medical university approved the experimental procedures (IACUC no. 20190129). Healthy adult Sprague-Dawley (SD) rats weighing 220 to 250 g were purchased from the Laboratory Animal Center of China Medical University (Shenyang, China). The rats were reared at 22 to 24 °C, with 40–60% relative humidity, a 12-h light-dark cycle, and ad libitum access to water and food. The rats (n = 60) were allocated randomly into three groups: A sham group (exposure of the sciatic nerve with no transection and the RF needle was placed but no energy was delivered), the SNI group (exposure of the sciatic nerve with partial transection and the RF needle was placed but no energy was delivered), and the PRF group (the exposure of the sciatic nerve with partial transection and the RF needle was placed and energy treatment was provided).

Behavioral tests

The rats were kept in separate metal mesh-bottomed, transparent cages, and allowed to acclimate for 2 h to test the basal paw withdrawal mechanical threshold (PWMT). The previously described up-down method, using von Frey hairs, was used to assess the PWMT. The first application comprised the 0.4 g stimulus, which was in the middle of the series. If a positive response (paw licking, shaking, or lifting) occurred within 5 seconds, a thinner filament was applied. In the case of a negative response, a thicker filament was used.

SNI model induction

Rat SNI surgery was executed following a previously described method. The rats were anesthetized via intraperitoneal injection of 10% chloral hydrate at 400 mg/kg. Then, an incision was made in the skin of the lateral thigh, followed by blunt dissection of the biceps femoris muscle to reveal the right sciatic nerve together with its three terminal branches (the tibial, common peroneal, and sural nerves). 5-0 silk was used to ligate the tibial and common peroneal nerves tightly. Then, transection of the nerves was performed distal to the ligation, removing a 2–4 mm section of the nerve fiber. Contact or stretching of the intact sural nerve was carefully avoided. The Sham group underwent this procedure without nerve injury. After surgery, the rats were returned to their original cages and environments.

Application of PRF
In the PRF group, a 21G PRF needle was inserted into the right L5 intervertebral foramen and placed in contact with the surrounding non-neural tissues. RF pluses were generated by the RF instrument (PMG-230, Baylis Medical Inc., Montreal, Canada) using the following settings: 2 Hz, 42 °C, and 50 V for 120 seconds. No energy was delivered through the needle in the sham and SNI groups. In a preliminary experiment we found that the peak of PWMT value and CCL2 expression were detected on day 7 after nerve injury; therefore, PRF treatment was administered on day 6.

**Specimen collection**

The rats were placed individually in a transparent sealed box for euthanasia. Isoflurane was administered into this box at 5 ml/kg and the rats were left into the box to breath in the isoflurane until they died (approximately 1–2 min). We isolated the l4-6 spine cord tissues from the rats, which were placed at -80 °C until western blotting analysis.

**Western blotting**

The spinal cord of the lumbar (L4-5) ipsilateral to the lesion was collected. Samples were ground in Radioimmunoprecipitation assay (RIPA) on ice. A BCA (bicinchoninic acid) Protein Assay kit (Pierce, Rockford, IL, USA) was used to determine the protein concentration. The samples were subjected to SDS-PAGE and then the separated proteins were electroblotted onto a polyvinylidene fluoride membrane.

5% Skimmed milk was used to block the proteins on the membrane for 2 h at room temperature. The membrane was washed using Tris buffered saline Tween (TBST) three times for 5 min each, before being incubated TBST-diluted primary antibodies at 4 °C overnight. The primary antibodies were anti-Phospho-NF-κB p65(Ser536) antibody (1:1000, 3303; Cell Signaling Technology, Danvers, MA, USA), anti-CCL2 antibody (1:2000, ab25124; Abcam, Cambridge, MA, USA), and anti-β-actin (1:4000, 20536-1-AP, Proteintech, Rosemont, IL, USA). The membranes were then washed in TBST (5min, three times) and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (1:10000, SA00001-2, Proteintech) for 2 h at room temperature. The membranes were washed again in TBST (10 min, three times), and then the immunoreactive proteins were visualized using electrochemiluminescence (ECL) reagents, and results were analyzed using the Gel Doc XR and ChemiDoc XRS system (Biorad, Hercules, CA, USA). The levels of the immunoreactive proteins were assessed quantitively using the grayscale values.

**Statistical analysis**

All statistical analyses were accomplished using SPSS 18.0 (IBM Corp., Armonk, NY, USA). All date are shown as the mean ± standard error. Repeated-measures analysis of variance (ANOVA) was used to analyze the PWMT. One way ANOVA was used to analyze the levels of CCL2 and phospho-NF-κB p65. Statistical significance was accepted at P < 0.05 was considered significant.

**Results**
Changes in behavior

Among the three groups, there were no significant difference in the PWMT before transection of the sciatic nerve. There were significant decreases in the PWMT score on day 1 (5.551 ± 0.934 vs. 0.795 ± 0.316), 3 (9.645 ± 1.686 vs. 0.319 ± 0.134), 7 (18.657 ± 2.788 vs. 0.240 ± 0.151), and 10 (17.904 ± 2.662 vs. 1.131 ± 0.454) after nerve injury in the SNI group compared with that in the sham group (P < 0.001). There were also significant decreases in the PWMT score on day 1 (5.551 ± 0.934 vs. 0.840 ± 0.332), 3 (9.645 ± 1.686 vs. 0.353 ± 0.135), 7 (18.657 ± 2.788 vs. 2.925 ± 0.754), and 10 (17.904 ± 2.662 vs. 6.923 ± 1.228) after nerve injury in the PRF group compared with that in the sham group (P < 0.01). No significant difference in the PWMT score was observed between the SNI and PRF group on day 0, 1, and 3 days in the absence of PRF treatment after sciatic nerve partial transection. After PRF application, there were significant increases in the PWMT score on day 7 (0.240 ± 0.151 vs. 2.925 ± 0.754) and 10 (1.131 ± 0.454 vs. 6.923 ± 1.228) in the PRF group compared with that in the SNI group (P < 0.05) (Fig. 1).

Western blotting analysis of CCL2 expression

After sciatic nerve section injury, the level of CCL2 increased immediately on day 1, 3, 7 and 10. The peak expression in the SNI group occurred at 7 days after nerve injury and was 2.46 times higher than that in the sham group (P < 0.05) (Fig. 2a). After PRF treatment, the level of CCL2 declined significantly in the PRF group compared with that in the SNI group. The relative protein level of CCL2 in the PRF group was 40.4% lower compared with that in the SNI group on day 7 (1 day after PRF) and was 66.2% lower than that in the SNI group on day 10 (4 days after PRF) (P < 0.05, Fig. 2b).

Western blotting analysis of NF-κB expression

After sciatic nerve section injury, the level of NF-κB increased significantly on days 3, 7, and 10. The peak expression in the SNI group occurred on day 7 after nerve injury and was 1.79 times higher than that observed in the sham group (P < 0.05) (Fig. 3a). After PRF treatment, the NF-κB levels decreased significantly declined in the PRF group. The relative protein expression of NF-κB in the PRF group was 25.6% lower than that in the SNI group on day 7 (1 day after PRF) and was 19.3% lower compared with that in the SNI group on day 10 (4 days after PRF) (P < 0.05, Fig. 3b).

Discussion

PRF alleviates hyperalgesia in SNI rats

In the present study, we demonstrated that after partial sciatic nerve transection, the PWMT values in the SNI group decreased significantly compared with those in the sham group. The SNI model can reliably simulate the clinical manifestations of NP in humans, for example hyperpathia, alldynia, and spontaneous pain. There was a linear decline in the PWMT value after nerve injury, comprising a rapid decline from the first day, reaching a minimum at 7 days, before rising slowly. Our findings are consistent with those of a previous study and indicated that the NP model was established successfully.
The most severe pain was observed at 7 days after partial nerve transection; therefore, PRF was applied to the DRG at 6 days after nerve injury. After 1 day of PRF treatment, there was a significant improvement in mechanical allodynia, which further improved after another 4 days. These results were similar to those of previous studies\textsuperscript{20,21}, which confirmed that PRF treatment of the DRG could alleviate hyperalgesia and allodynia significantly in an NP model.

The DRG is found at the upper region of the intervertebral foramen, which contains the primary class of sensory neurons and glial cells. After nerve injury, the activated neurons and glial cells in the DRG release many kinds of inflammatory cytokines, which are involved in nociceptive transmission and central sensitization. The DRG has an important role in NP development and maintenance, making it an excellent clinical target for chronic pain treatment\textsuperscript{22,23}. Therefore, in the present study, the PRF current duration was applied on the L4-5 DRG to achieve excellent pain relief.

**CCL2 and NF-κB levels increased in the SNI model**

CCL2 is constitutively expressed in neurons and glial cells, and is dramatically upregulated in the early stage of peripheral nerve injury\textsuperscript{24,25}. Nuclear factor kappa B (NF-κB) is a key transcription factor that regulates the expression of many genes (e.g., those encoding cytokines, chemokines, and neural nitric oxide synthase (nNOS)) that are important in NP development\textsuperscript{26,27}. After being phosphorylated and activated in response to cell stimulation, NF-κB acts as a critical element in CCL2 induction\textsuperscript{28}. In the present study, we found that CCL2 and NF-κB levels in the spinal cord increased immediately after sciatic nerve partial transection. The levels of CCL2 and NF-κB both peaked at 7 days after nerve injury and then gradually declined, which indicated that CCL2 expression might be regulated primarily by NF-κB. These results were in agreement with the results of Gao\textsuperscript{29}, who found that the increase in CCL2 expression could be observed from days 1 to 10, and peaked at day 7 in the spinal cord in an SNI model.

**PRF might exert an analgesic function by reducing CCL2 levels in the spinal cord**

Western blotting analysis revealed that the CCL2 contents in the spinal cord of the PRF group decreased significantly on day 7 and 10 compared with those in the SNI group. The trend of NF-κB levels was the same as that for CCL2 in the SNI and PRF groups. NF-κB is also a critical upstream regulator of CCL2 expression. These results suggested that PRF reduced CCL2 levels by inhibiting the phosphorylation and activation of NF-κB in the rat spinal cord. CCL2 has a low expression levels in neurons and glial cells under normal conditions. Once peripheral nerve injury occurs, CCL2 could be released immediately, which would lead to glial cell activation, ultimately causing the release of several pain regulators, including nerve growth factor, interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and CCL2\textsuperscript{30,31}. These pain regulators are involved in central sensitization and lead to the development and maintenance of NP\textsuperscript{32}. According to our experimental results, we hypothesized that PRF inhibits this chain reaction by downregulating the expression of CCL2, which might represent one of the mechanisms by which PRF treatment achieves analgesia.
Limitations

The results of the present study demonstrated that PRF could reduce hyperalgesia and decrease CCL2 and NF-κB level in the spinal cord in the early stage of NP in the SNI model; however, the detailed relationships among hyperalgesia, CCL2, and PRF require further research. We observed the changes of pain threshold, and CCL2 and NF-κB levels only up to 4 days after PRF administration; thus, to determine the long-term efficacy of PRF, longer follow-up is required. We only examined the expression of CCL2 in the spinal cord, but not in other parts, including the DRG and the dorsal horn of the spinal cord. Whether PRF has the same treatment effect in the presence of antagonists of CCL2 or CCR2 also needs further experimental clarification.

Conclusions

PRF effectively alleviated hyperalgesia after administrated to dorsal root ganglion in early stage after nerve injury. The mechanism might reduce the expression of the CCL2 though inhibiting NF-κB activation in the spinal dorsal horns, which would then prevent the transmission of pain signals to the CNS.

Declarations

Funding statement

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Authors' contributions

Cheng-fu Wan performed the majority of the experiments, designed the study and acquired data, was involved in the analysis and interpretation of data, participated in drafting the manuscript, in critical revision of important content and gave final approval of the version to be released.

Bo-han Zhang and Dao-song Dong contributed to data interpretation and analysis. Tao Song made a substantial contribution to the experimental design, drafting and critically revising the manuscript. All of the authors have read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were conducted in accordance with the Guiding Principles for research involving animal and Human Beings, and the experimental procedures were approved by the animal care and use
committee of china Medical university (iacuc no. 20190129).

**Patient consent for publication**

not applicable.

**Competing interests**

All the authors declare that they have no competing interests.

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**Figures**

![Figure 1](image-url)
The change of PWMT after sciatic nerve section injury; *P<0.001, sham group vs. SNI group; #P<0.01, sham group vs. PRF group; +P<0.05, SNI group vs. PRF group. Data are expressed as the mean±standard error.

Figure 1

The change of PWMT after sciatic nerve section injury; *P<0.001, sham group vs. SNI group; #P<0.01, sham group vs. PRF group; +P<0.05, SNI group vs. PRF group. Data are expressed as the mean±standard error.
Figure 2

2a: The relative protein expression of CCL2 in spinal cord after sciatic nerve section injury. ∗P<0.05, sham group vs. SNI group. Data are expressed as the mean±standard error. 2b: The relative protein expression of CCL2 in the spinal cord on 7 and 10 days after sciatic nerve section injury. ∗P<0.05, SNI group vs. PRF group. Data are expressed as the mean±standard error.
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Figure 3

3a: The relative protein expression of NF-κB in spinal cord after sciatic nerve section injury. *P < 0.05, sham group vs. SNI group. Data are expressed as the mean ± standard error. 3b: The relative protein expression of NF-κB in spinal cord on 7 and 10 days after PRF. *P < 0.05, SNI group vs. PRF group. Data are expressed as the mean ± standard error.
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