Pulsed radiofrequency alleviated neuropathic pain by down-regulating the expression of substance P in chronic constriction injury rat model

Jing-Ai Wang[1], Shao-Ning Niu[2], Fang Luo[3]

[1]Department of Anesthesiology, Plastic Surgery Hospital, Chinese Academy of Medical Science, Beijing 100144, China;
[2]Department of Anesthesiology, Beijing Ditan Hospital, Capital Medical University, Beijing 100015, China;
[3]Department of Pain Management, Beijing Tiantan Hospital, Capital Medical University, Beijing 100050, China.

Abstract

Background: Pulsed radiofrequency (PRF), as a non-invasive treatment of neuropathic pain (NP), has been widely administered clinically. Previous studies have shown that PRF has the potential to improve hyperalgesia in animal models of NP. However, there have been few reports to clarify whether the mechanism of PRF treatment of NP involves intervention in the expression of substance P (SP). Therefore, this study administered PRF treatment to chronic constriction injury (CCI) model rats and observed the sciatic nerve mechanical pain threshold and SP expression in the spinal cord to explore the mechanism of PRF treatment.

Methods: A total of 96 Sprague-Dawley rats were randomly divided into the sham-surgery-sham-treatment group (S-S group), the sham-surgery-PRF group (S-P group), the CCI-sham-treatment group (C-S group), and the CCI-PRF group (C-P group). The C-S group and the C-P group underwent sciatic nerve CCI, while the other groups received a sham operation. At 14 days after the operation, the C-P group and the S-P group were treated with PRF for 300 s. We recorded the hindpaw withdrawal threshold (HWT) and the thermal withdrawal latency (TWL) of rats in the various groups at baseline, before treatment (0 days), and at 1, 7, 14, and 28 days after treatment. L4 to L6 spinal cord tissues were taken before treatment (0 days) and 1, 7, 14, and 28 days after treatment. The transcription and translation of SP were measured by quantitative polymerase chain reaction and Western blotting, respectively.

Results: The HWT and the TWL in the C-P group 28 days after PRF treatment were significantly higher than those in the C-S group (95% confidence interval [CI]: 5.84–19.50, $P < 0.01$; 95% CI: 2.58–8.69, $P = 0.01$). The expression of SP in the C-P group 28 days after PRF treatment was significantly lower than that in the C-S group (95% CI: 1.17–2.48, $P < 0.01$).

Conclusions: PRF may alleviate CCI-induced NP by down-regulating the expression of SP in the spinal cord of CCI model rats.

Keywords: Pulsed radiofrequency; Chronic constriction injury; Sciatic nerve; Substance P

Introduction

The current definition of neuropathic pain (NP) according to the International Association for the Study of Pain is "pain initiated or caused by a lesion or dysfunction in the somatosensory system."[1] Treatments for NP differ from other types of pain[2] due to the risk of permanent loss of function caused by neurological impairment. The therapeutic effect of anti-epileptic drugs is limited, and long-term application of these drugs can even lead to unintentional side effects.[3] Refractory cases that respond poorly to drug treatment are likely to undergo interventional treatment or surgery in the clinic. Pulsed radiofrequency (PRF) treatment, which was first proposed by Sluijter,[4] is a non-destructive, minimally invasive technique applied to the nervous system, and it has recently become an increasingly popular technique for the clinical treatment of NP.[5-7] Contrast to the continuous high frequency current emitted by conventional continuous radiofrequency, the radiofrequency current generated by PRF lasts only 20 ms per stimulus, followed by an inter-stimulus interval of 480 ms, allowing the complete dissipation of heat generated by the current near the target tissue and preventing nerve damage caused by excessive heat. However, the mechanism of PRF-mediated treatment of NP remains unclear.

Recent studies[8,9] suggest that neurological impairment in NP patients can cause immune cells and immune-like glial cells to release proinflammatory cytokines, chemokines,

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Jing-Ai Wang and Shao-Ning Niu contributed equally to this work.

Correspondence to: Dr. Fang Luo, Department of Pain Management, Beijing Tiantan Hospital, Capital Medical University, Beijing 100050, China
E-Mail: luofangwt@yahoo.com

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and other factors that mediate pain, such as substance P (SP), calcitonin gene-related peptide, and glutamate in injured nerves, dorsal root ganglia (DRG), and the spinal cord. Subsequently, an immune system response is stimulated, which participates in pain signaling. During NP occurrence and development, SP seems to play a vital role as a neuropeptide that mediates pain signal transmission *in vivo*.[10]

Previous studies have confirmed that PRF can improve hyperalgesia in NP model rats,[11-14] while the expression levels of factors related to the transmission of pain signals in rats, such as glutamate, insulin-like growth factor, and extracellular signal-regulated kinase, are significantly decreased.[11,15,16] However, whether the mechanism underlying PRF-mediated treatment of NP involves interfering with the expression of SP has not been reported. The aim of this study was to investigate the analgesic effect of PRF acting directly on the sciatic nerve ligation site of chronic constriction injury (CCI) model rats and the resulting changes in SP expression in the spinal cord. We also sought to identify a mechanistic target for future studies aimed at improving the efficacy of PRF.

Methods

Experimental animals and groups

Ninety-six healthy male Sprague-Dawley rats aged 4 months with a body mass of 200 to 220 g were provided by the Beijing Vital River Laboratories and were quarantined for 1 week at the Laboratory Animal Center (specific pathogen-free level) of Tiantan Hospital Affiliated to Capital Medical University.

The 96 rats were randomly divided into the sham-surgery-sham-treatment group (S-S group, $n = 24$), the sham-surgery-PRF group (S-P group, $n = 24$), the CCI-sham-treatment group (C-S group, $n = 24$), and the CCI-PRF group (C-P group, $n = 24$).

CCI model preparation

CCI was developed in the C-S group and the C-P group. Anesthesia was induced by an intraperitoneal injection of 10% chloral hydrate at 400 mg/kg. The rats were placed in the prone position, and the right hind limb skin was prepared and subjected to routine disinfection. A skin incision was made lateral to the middle line, and blunt separation was conducted along the lateral biceps femoris to expose the sciatic nerve trunk. Four circular suture ligations were made in the proximal side of the sciatic nerve trifurcation using a 4-0 chromic gut suture, with an interval of 1 mm between ligations, and the strength was just sufficient to cause mild twitching of the calf muscle.[17] At the end of the surgery, the incision was sutured layer by layer, and the rats were returned to their original housing environment.

Intervention methods

The sciatic nerve was exposed again at 14 days after the sciatic nerve ligation/sham operation in the C-P group and the S-P group. PRF treatment was administered at the site of sciatic nerve ligation or at the corresponding site in the sham operation group. The therapy instrument (PMG-230, Baylis Medical Inc., Montreal, Canada) was used in manual PRF mode. The parameters included a pulse frequency of 2 Hz, a temperature of 42°C, an output voltage of 45 V, and a duration of 300 s. During the course of treatment, we observed slight twitching in the right thigh of the rat. The sciatic nerve was exposed again at 14 days after sciatic nerve ligation/sham operation in the C-S group and the S-S group, and an unenergized electrode needle was placed at the site of sciatic nerve ligation or at the corresponding site in the sham operation group.

Determination of the mechanical pain threshold

Six rats were randomly selected from each group, and the hindpaw withdrawal threshold (HWT) and the thermal withdrawal latency (TWL) were measured. The rats were placed in cages with a metal mesh bottom that were separated from each other and were pre-conditioned for at least 1 h. Von Frey hairs of different pressures were used to perpendicularly stimulate the right posterior plantar of the rats. Stimulation was delivered five times for each pressure. Each time, the probe was slightly bent and applied for 3 to 5 s at intervals of 30 s. If more than three out of five stimulation trials induced the foot flinching reflex, the HWT of this foot was considered to be triggered.[18] The pressure was initialized at 1 g and increased in intervals according to 2, 4, 6... 60 g until the foot flinching reflex was detected, at which point the threshold was recorded. The baseline value of the pain threshold was measured before the model/sham operation.

The rats were then placed in a transparent box, and Hargreaves method[19] was employed to measure the TWL values. The heat center of the mobile infrared radiant heat stimulus generator (Ugo Basile 37370, Italy) was focused on the right hindpaw of the rats. Persistent radiation was applied to the right hindpaw, and the latent time between the start of infrared radiant heat and the reflective retraction was recorded as the TWL. The test was repeated three times and the average value was recorded, although the interval of heat stimulation never exceeded 2 min to avoid tissue damage.

The HWT and TWL tests were performed 14 days after the model/sham operation, that is, before treatment/sham treatment (0 days) and at 1, 7, 14, and 28 days after treatment/sham treatment.

Quantitative polymerase chain reaction (qPCR) detection

Six rats were randomly taken from each group before treatment/sham treatment (0 days) and at 1, 7, 14, and 28 days after treatment/sham treatment. The rats were sacrificed by decapitation, and the L4 to L6 spinal cord was quickly extracted and stored in liquid nitrogen. All tissues were divided into two equal parts, and one set of tissue samples was used for qPCR. Total RNA was extracted using the Invitrogen Trizol method. The extracted RNA was reverse-transcribed to obtain the first strand of complementary DNA according to the instructions of the NEB MMLV First Chain Synthesis Kit. Based on the gene sequence information in GenBank, the primer design software Primer3

Table 1: Primer and internal reference gene expression sequences.

Gene	GenBank number	Product length (bp)	Primer	Sequences (5'-3')
Tac1	NM_012666	116	Forward	TGATCTAAATTATTGGTCCGACTG
			Reverse	CAAAGAACTGCTGAGGCTTGG
β-actin	NM_031144	150	Forward	CCCATCTATGAGGGTTACG
			Reverse	TTTAATGTCACGCACGATT

(PREMIER Biosoft Inc., San Francisco, CA, USA) was used to design primers suitable for the SYBR green method. β-actin was used as the internal reference gene [Table 1]. Analysis was conducted using a real-time fluorescence qPCR system (ABI StepOnePlus, Applied Biosystems, Waltham, MA, USA). The reliability of the qPCR reaction was judged based on the melting curve and the standard curve, and the measured threshold cycle number (Ct) was then obtained. The difference was compared to the Ct value of the internal reference β actin gene; that is, ΔCt (delta Ct mean) can be used to calculate the ΔΔCt value, and the relative levels of gene expression were then compared between the samples based on the $2^{-\Delta\Delta Ct}$ formula.

Western blotting detection

At each time point, another set of samples was taken from each group for Western blotting detection. The tissue was homogenized until full lysis and then centrifuged for 5 min at 12,000 × g. The supernatant was extracted, and the protein concentration was determined according to the instructions of the bicinchoninic acid protein concentration assay kit (Sigma-Aldrich, St. Louis, MO, USA). The protein concentration of the protein extract from each group of tissue was adjusted, and the protein solution was mixed with two volumes of loading buffer to generate the sample loading solution. We denatured and centrifuged the sample loading solution, which was then subjected to 30 min of 70 V constant voltage electrophoresis; this step was followed by 90 V constant voltage electrophoresis when the indicator bromothymol blue entered the separation gel. Immediately before the termination of electrophoresis, a polyvinylidene fluoride (PVDF) membrane was immersed in methanol for 15 s, then rinsed in double distilled H_2O for 2 min, and finally soaked in the transfer buffer for 5 min before the subsequent procedures were started. The electric membrane transfer instrument was placed in ice water, and membrane transfer was conducted at a 200 mA constant current for 70 min. After the membrane was transferred, the PVDF membrane was quickly removed and blocked in 5% bovine serum albumin at room temperature for 2 h, followed by three washes of 5 min each. The tris-buffered saline tween (TBST) diluted antibody (SP antibody from Bioss, catalog number bs-0065R, dilution ratio 1:1000) was added to the incubation chamber and incubated overnight at 4°C. The membrane was washed with TBST for 5 min × 3 times and then incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody 2 h at room temperature, followed by washing with TBST for 10 min × 3 times. The membrane was finally incubated in the chemiluminescence detection reagent (reagent A:reagent B = 1:1) for 2 min.

Statistical analysis

SPSS 20.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis, and GraphPad Prism 6.0 was used to generate graphs. Measurement data with normal distributions were expressed as mean ± standard deviation. HWT and TWL results were compared using variance analysis for repeated measurements and multiple comparisons using the Bonferroni method. The expression levels of SP measured by qPCR and Western blotting were compared by one-way analysis of variance and *post hoc* analysis using the least significant difference (LSD) method. A $P < 0.05$ indicates that the difference was statistically significant, and $P < 0.01$ indicates that the difference was very significant.

Results

Changes in HWT and TWL

There was no significant difference in baseline HWT and TWL levels among the four groups ($P > 0.05$). The HWT and TWL values in the C-S group and the C-P group at 14 days after modeling were significantly lower than those in the S-S group and the S-P group ($P < 0.01$).

At 1 day after PRF treatment, the HWT value of the C-P group showed a slight increase, but there was no significant difference in the HWT between the C-P group and the C-S group (95% confidence interval (CI): −2.05 to 10.35; $P = 0.385$). At 7 days after PRF treatment, the HWT value of the C-P group was significantly higher than that of the C-S group (95% CI: 3.59–14.91; $P = 0.01$). At 14 and 28 days after PRF treatment, the HWT values of the C-P group showed highly significant differences from the HWT values of the C-S group (95% CI: 0.28–17.62; $P < 0.01$, 95% CI: 5.84–19.50; $P < 0.01$) and no significant differences from the HWT values of the S-S group (95% CI: −1.82 to 9.52; $P = 0.364$, 95% CI: −2.92–6.82; $P = 0.413$) [Figure 1].

The changes in the TWL were similar to the HWT as described above, and the TWL value of the C-P group began to increase after 1 day of treatment. The TWL value of the C-P group was significantly higher than that of the C-S group (95% CI: 2.67–6.79; $P < 0.01$) at 7 days after PRF treatment, and the increasing trends continued through day 28 (95% CI: 2.58–8.69; $P = 0.01$). There were no significant differences in the TWL between the C-S group and the S-S group ($P > 0.05$) [Figure 2].

qPCR evaluation of SP mRNA changes

The expression of SP mRNA in the spinal cord of the C-P group and the C-S group was significantly higher than that

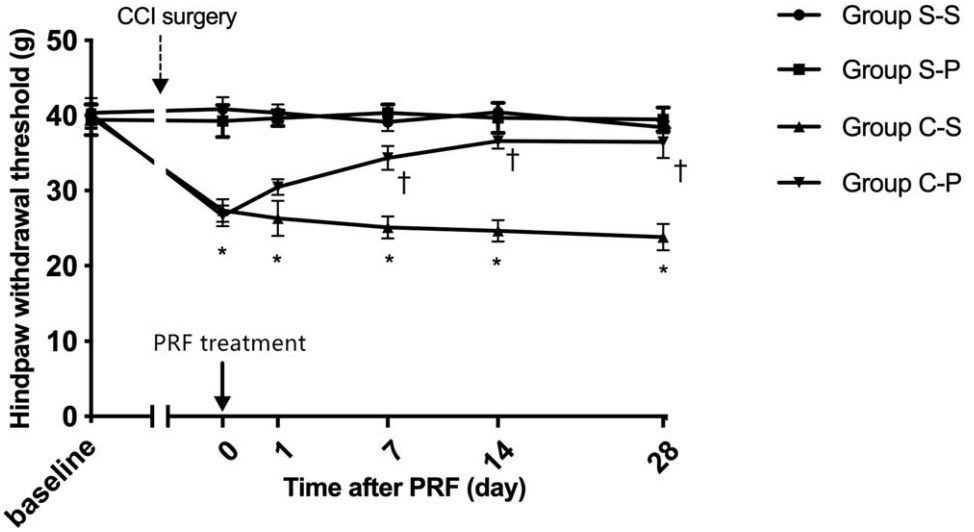

Figure 1: Changes in HWTs in rats. *Comparison between group S-S and group C-S ($P < 0.01$); †Comparison between group C-P and group C-S ($P < 0.01$). CCI: Chronic constriction injury; HWT: Hindpaw withdrawal threshold; PRF: Pulsed radiofrequency.

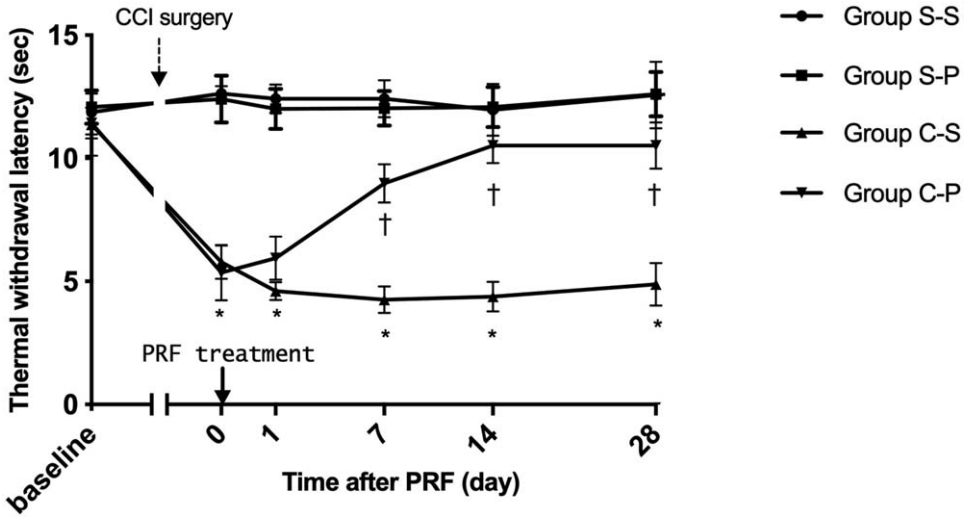

Figure 2: Changes in TWLs in rats. *Comparison between group S-S and group C-S ($P < 0.01$); †Comparison between group C-P and group C-S ($P < 0.01$). CCI: Chronic constriction injury; PRF: Pulsed radiofrequency; TWL: Thermal withdrawal latency.

in the S-P group and the S-S group ($P < 0.01$) at 14 days after modeling, that is, before PRF treatment/sham treatment (0 days). At 1 day after treatment/sham treatment, there was no significant difference between the C-P group and the C-S group (95% CI: −0.83 to 0.10; $P = 0.115$). The expression of SP mRNA in the C-P group at 7 days after treatment was significantly lower than that in the C-S group (95% CI: 0.32–1.66; $P < 0.01$), and the expression of SP mRNA in the C-P group was further reduced at 14 and 28 days after treatment to a level significantly lower than that in the C-S group with very significant differences (95% CI: 0.86–2.23; $P < 0.01$, 95% CI: 1.17–2.48; $P < 0.01$). In contrast, the expression of SP mRNA in the C-S group remained at a higher level until day 28 and showed very significant differences compared with the levels in the S-S group and the S-P group ($P < 0.01$) [Figure 3].

Western blotting evaluation of SP protein expression changes

Consistent with the qPCR results, the expression of SP protein was significantly higher in the spinal cord of the C-S group and the C-P group than in the S-S group and the S-P group ($P < 0.01$) at 14 days. The expression of SP protein in the C-P group began to decline at 1 day after treatment compared with the C-S group. The difference between the two groups became significant at 14 and 28 days after treatment (95% CI: 0.89–2.23; $P < 0.01$, 95% CI: 0.64–1.79; $P < 0.01$). The expression level of SP

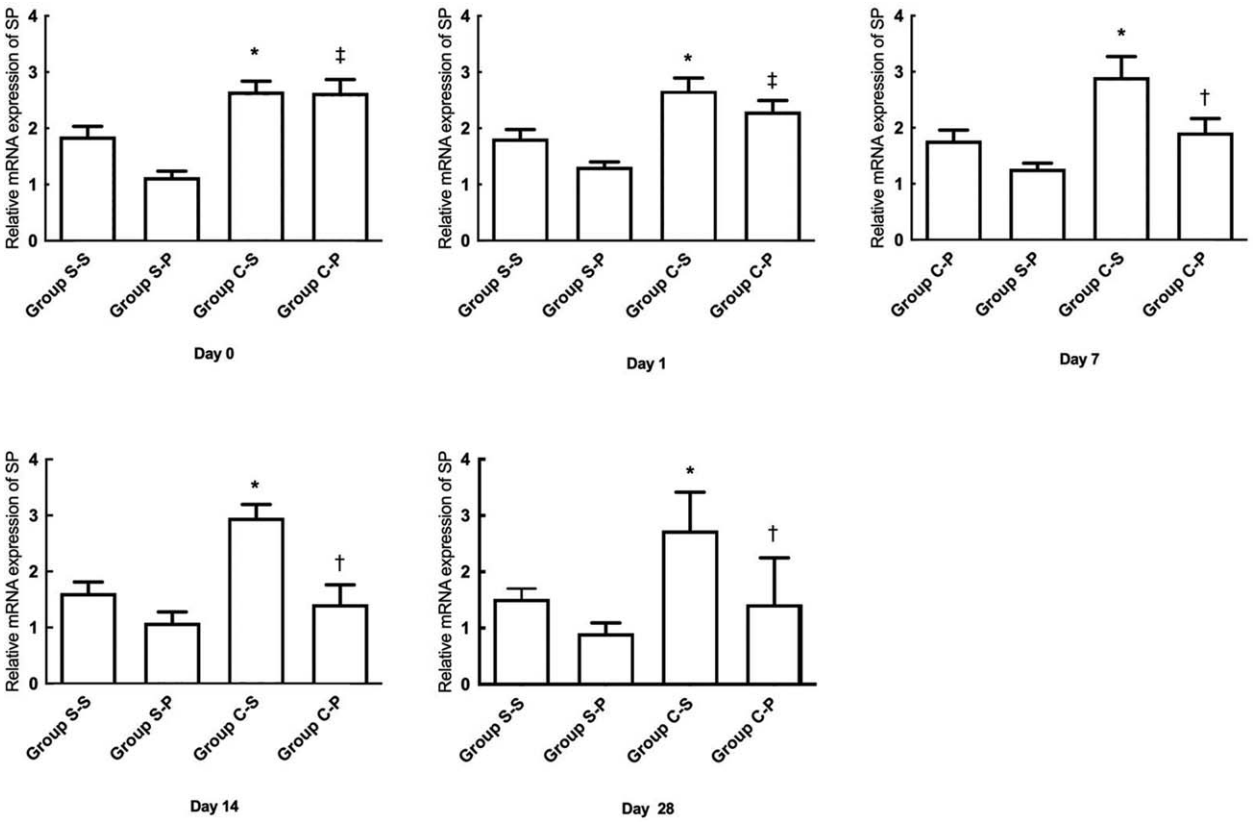

Figure 3: The mRNA expression of SP in the spinal cord. *Comparison between group S-S and group C-S ($P < 0.01$); †Comparison between group C-P and group C-S ($P < 0.01$); ‡Comparison between group C-P and group S-S ($P < 0.01$). SP: Substance P.

protein in the C-S group remained at relatively higher levels and consistently showed significant differences from the expression levels in the S-S group and the S-P group ($P < 0.01$) [Figure 4].

Discussion

In this study, the HWT value in the CCI rat model after sciatic nerve ligation was significantly lower than that of the untreated rats, indicating successful simulation of mechanical hyperalgesia caused by NP. PRF was directly applied to the sciatic nerve ligation site of rats. The HWT value of the C-P group at 7 days was significantly higher than before treatment and significantly higher than that of the C-S group, which underwent sham treatment, at 7 days. The HWT value of the C-P group continued to increase at 14 days and even 28 days after PRF treatment. Thus, consistent with previous studies,[20,21] this study demonstrates that PRF can significantly relieve mechanical hyperalgesia in CCI model rats.

Consistent with previously reported findings,[12,13] hyperalgesia in the CCI model did not show immediate remission after PRF treatment but did progressively improve, which may be associated with the special nerve conditioning mechanism by which PRF treats NP. PRF-mediated nerve conditioning may lead to a series of changes in the pain transduction pathway, gradually achieving the effect of alleviating NP. This gradual onset of treatment efficacy is also consistent with the clinical phenomenon that satisfactory pain relief is gradually achieved after PRF treatment of NP.[22-24] After PRF treatment of NP, clinicians should conduct individualized dose adjustment of drugs, such as anti-epileptic drugs, based on changes in the patient's pain. Importantly, clinicians should take care to wait a sufficient amount of time for the onset of treatment efficacy, and they should avoid prematurely concluding that PRF treatment is invalid while moving to more invasive treatment methods. Nevertheless, clinicians should not wait blindly but should understand the onset time range of PRF treatment. If PRF treatment is considered to be invalid, other treatment methods should be applied to reduce the pain of patients.

According to our study, there was no significant change in the HWT or the TWL after PRF treatment in the S-P group without sciatic nerve ligation, indicating that PRF treatment itself did cause injury to healthy sciatic nerve or cause mechanical hyperalgesia. Consistent with previous studies,[14,25,26] this study confirms that PRF is a safe, non-destructive, and minimally invasive technique for treating a nervous system. Although PRF itself induces no side effects of nerve damage, clinicians must certainly focus on the post-operative response to the puncture technique, especially when punctures are difficult and when repeated punctures are required.

In this study, the expression of SP was significantly increased at the gene and protein expression levels in the spinal cords of model rats. In recent years, many

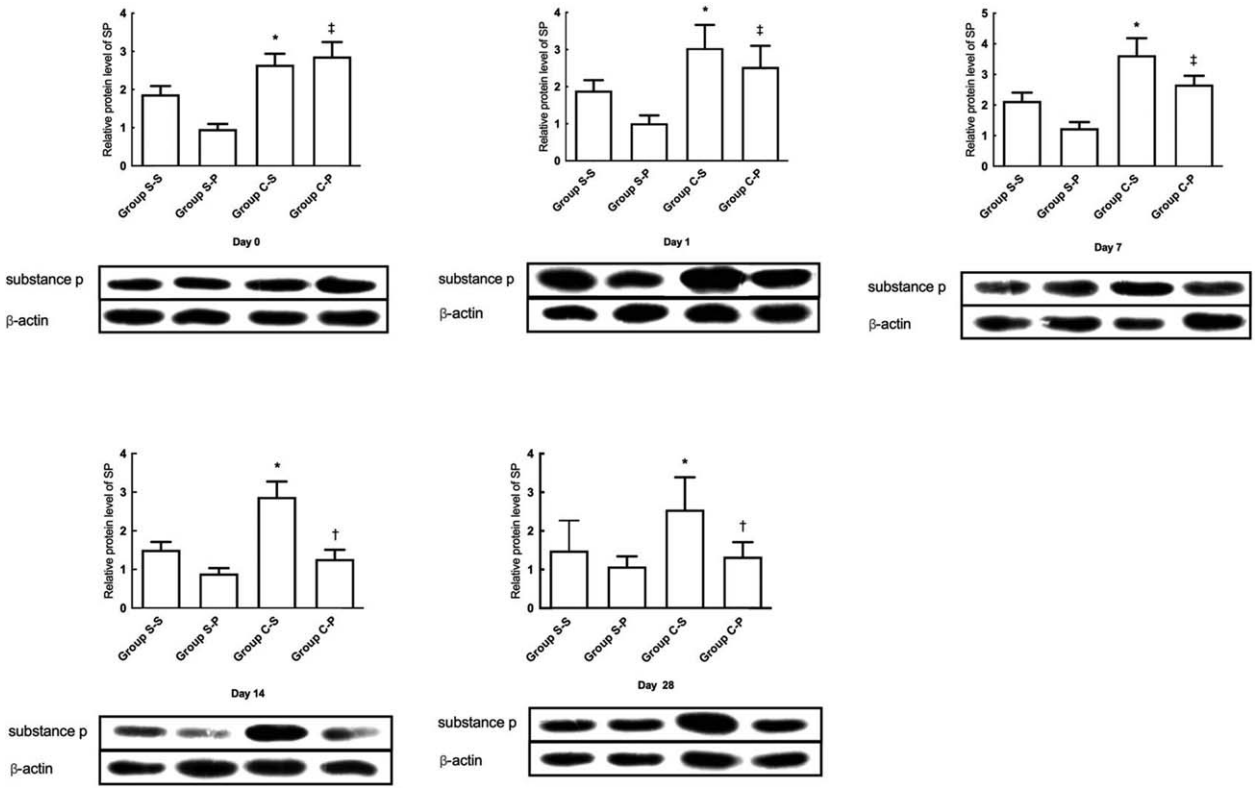

Figure 4: Representative Western blotting and relative SP expression in the spinal cord. *Comparison between group S-S and group C-S ($P < 0.01$); †Comparison between group C-P and group C-S ($P < 0.01$); ‡Comparison between group C-P and group S-S ($P < 0.01$). SP: Substance P.

studies[27,29] have confirmed that the endogenous neuropeptide SP plays a role in mediating the transduction of nociceptive information in the human body. In an infraorbital nerve CCI rat model, it was also observed that the occurrence and development of NP were associated with an increase in SP expression,[28] suggesting that the accumulation of SP may be associated with ectopic nerve activity, which in turn leads to NP. Nociceptive signals stimulate the primary afferent nerve and activate peptidergic neurons in the DRG, causing the release of SP and the binding of SP to its specific receptor, neurokinin-1 (NK-1), which sensitizes neurons and produces pain sensation and other biological effects.[29] A significant increase in SP levels was found in the spinal cord of the rat model of peripheral NP, suggesting that SP may be involved in the occurrence of peripheral NP.[30] A prolonged noxious afferent input may initiate the sensitization of spinal dorsal horn cells and facilitate spinal reflexes. In addition, synaptic transmissions were enhanced by long-term potentiation (LTP). Glutamate and tachykinin receptors are involved in the transition to LTP, and LTP could be prevented by administration of NK-1 receptor antagonists.[31] Biochemical analysis[32] confirmed that SP mRNA and protein expression levels are significantly increased in the NP rat lumbar spinal cord. Starnowska *et al* proposed that the administration of opioid agonist/NK-1 receptor antagonist hybrid peptides via lumbar puncture to the lumbar spinal cord of the CCI model could induce a certain analgesic effect. This finding may suggest that pharmacological blockade of neuropeptide substances that affect the transmission of pain signals can relieve the hyperalgesia caused by nerve injury, thereby providing a more precise and effective targeted therapy. An animal experiment[33] confirmed that simultaneous injection of an NK-1 receptor antagonist into local areas and into the nerve sheath of the rat NP model can produce an effective analgesic effect. Therefore, reducing SP expression may be a viable option for targeted NP therapy.

A recent study[34] found that docosahexaenoic acid treatment can significantly reduce SP and nitric oxide-like neurotransmitter expression levels in the rat NP model and reduce astrocyte hyperplasia in the shallow lamina of the spinal cord dorsal horn, thereby reducing the pain symptoms in NP, which confirms the correlation between a reduction in SP levels and a reduction in NP symptoms. In this study, the pain in CCI model rats was gradually relieved after PRF treatment, while the SP translation and protein expression levels in the spinal cord also decreased gradually. In contrast, at 2 weeks after PRF treatment, the C-S group, which received sham treatment, showed no remission in the HWT, and SP expression in the spinal cord was maintained at a level higher than in the control group. Our results suggest that the mechanism by which PRF reduces the HWT and the TWL in the NP model may be related to the down-regulation of SP expression.

The analgesic effect of PRF is thought to be related to changes in generated electric fields, rather than changes in temperature or magnetic fields.[25] It has been suggested

that PRF-produced electric fields with rapid and continuous changes can exert neuronal regulation at the molecular and cellular levels. Animal experiments also confirmed that PRF treatment could activate the neurons in the DRG and the spinal dorsal horn that control pain signal.[35] Therefore, we speculate that PRF electric field effects may lead to the down-regulation of SP expression. In the future, confirming whether the electric field effect at different intensities is related to changes in SP expression and pain behavior would provide a theoretical basis for improving the treatment efficacy of PRF.

This study only explored whether the mechanism of PRF treatment in the rat sciatic nerve CCI model may involve the down-regulation of SP expression. Whether the mechanisms of PRF treatment in other NP models are also related to the expression of SP remains to be further investigated. In the future, determining whether the effect of PRF is observed after applying the antagonist of the SP-specific receptor NK-1 would further confirm whether the mechanism of PRF treatment of NP is related to changes in SP expression. Therefore, the lack of antagonistic groups is a limitation of this study. A published study[36] confirmed that significant effects of PRF treatment were observed at 14 days after treatment. The endpoint of our study was 28 days after treatment, and thus, the longer-term efficacy of PRF still needs to be explored. Another recent study[37] showed that the DRG are deeply involved in peripheral processes that lead to NP, although our study only measured the translation and transcription levels of SP in the spinal cord. However, SP expression changes in other parts of the nociceptive sensory pathway, such as in the DRG and the sciatic nerve, were not measured. PRF treatment of NP may involve a variety of neuropeptide substances; thus, the fact that this study only measured the expression level of one neuropeptide, SP, is a limitation.

In conclusion, the use of PRF at the sciatic nerve ligation site of CCI model rats can safely and effectively relieve mechanical hyperalgesia due to NP caused by CCI. The therapeutic mechanism may involve the inhibition of SP expression in the spinal cord.

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Conflicts of interest

None.

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