Original Research Article

Pulsed Radiofrequency Application Reduced Mechanical Hypersensitivity and Microglial Expression in Neuropathic Pain Model

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Abstract

Objective. Pulsed radiofrequency (PRF) procedure has been used in clinical practice for the treatment of chronic neuropathic pain conditions without neuronal damage. The purpose of this study was to investigate the changes in pain response and glial expression after the application of PRF on a dorsal root ganglion (DRG) in a neuropathic pain model.

Design. A neuropathic pain model (14 female Sprague-Dawley [SD] rats; 200–250 g) was made by a unilateral L5 spinal nerve ligation (SNL) and transection on the distal side of the ligation. The development of mechanical and cold hypersensitivity on the hindpaw was established postoperative day 9 (POD 9). The rats were then randomly assigned to the PRF (+) and the PRF (−) groups. Furthermore, PRF (2 bursts/s, duration = 20 milliseconds, output voltage = 45 V) was applied on the ipsilateral DRG for 180 seconds, with a maximum temperature of 42°C, at POD 10. Pain behaviors were tested throughout the 12 days after PRF. We also examined the changes of the spinal glial expression by immunohistochemistry.

Results. Significant reduction of mechanical hypersensitivity in the PRF (+) group was observed from day 1 after a single PRF procedure and was maintained throughout the following 12 days. Immunoreactivity for OX42 in the ipsilateral dorsal horn also decreased compared with that of the PRF (−) group. However, cold hypersensitivity and glial fibrillary acidic protein (GFAP) immunoreactivity in the dorsal horn was not affected by a PRF procedure.

Conclusions. Our result demonstrated that the mechanical hypersensitivity, induced by L5 SNL, was attenuated by a PRF procedure on the ipsilateral DRG. This analgesic effect may be associated with an attenuation of the microglial activation in the dorsal horn.

Key Words. Pulsed Radiofrequency; Neuropathic Pain; Mechanical Hypersensitivity; Microglia; Spinal Nerve Ligation

Introduction

Neuropathic pain, caused by an injury to the peripheral nerve or central nervous system (CNS), is the state of being in chronic pain and is a challenging condition to treat. Despite the various trials of pharmacological drugs, medical treatment of neuropathic pain is still unsatisfactory. The glial activation and neuron to glial interaction, via neurotransmitters and neuromodulators in the spinal...
dorsal horn, are involved in the development of neuropathic pain [1,2]. The modulation of the glial function and signaling to the neuron in the spinal cord is an emerging therapeutic strategy for the treatment of neuropathic pain [3].

Radiofrequency (RF) has been used for chronic pain management in clinical practice for more than 30 years [4,5]. There are two types of RF procedures that are used for continuous and pulsed stimulation. In particular, the modified technique, pulsed radiofrequency (PRF) procedure, delivers short pulses of RF energy to the dorsal root ganglion (DRG) or other nervous tissue. It has a long-lasting analgesic effect and is used as a minimally neurodestructive technique for the treatment of chronic pain. It seems to have an effect on chronic pain transmission via a neuromodulatory effect, rather than a thermal lesioning of the nervous tissue, when compared with that of the continuous radiofrequency (CRF) [6]. Chronic pain syndromes, treated by PRF, include lumbar radicular pain [7,8], cervicogenic headache [9], postherpetic neuralgia [10], peripheral neuralgia [11], and phantom pain [12].

Although PRF has been used clinically for a long time, the mechanism of the analgesic effect of PRF to neuropathic pain has not been fully understood. PRF has been reported to induce acute and late changes of cellular activities and gene expression in the spinal dorsal horn or DRG [13–15]. Their cellular activities are selective in the neurons with small diameter nerve fibers [13]. However, there is a lack of studies on the effects of PRF and its analgesic mechanism in neuropathic pain conditions. We investigated the changes in pain behavior and neuroglial expression in the spinal dorsal horn after an application of PRF on DRG in a neuropathic pain model.

Materials and Methods

Animals

Female Sprague-Dawley rats (N = 14, 200–250 g) were used in this study. All experimental procedures were carried out according to the guidelines of the Institutional Animal Care and Use Committee at Yeungnam University, South Korea.

Neuropathic Pain Model

A neuropathic pain model was made by L5 spinal nerve ligation (SNL) and transection on the distal side of the ligation, which was modified from the Chung model [16]. Briefly, rats were anesthetized with Zoletil (50 mg/kg, i.p; Virbac Laboratories, Carros, France). Under sterile conditions, a longitudinal incision, at the lower lumbar and sacral level, as well as the separation of paraspinous muscles, was made in order to expose the right L6 transverse process. Following the removal of the right L6 transverse process, by a small rongeur, the exposed L5 spinal nerve was isolated and tightly ligated with a piece of 6-0 silk thread, and was then cut just distal to the ligation, in order to interrupt all the axons. Following the surgery, the incised muscles and skin were sutured in layers, and rats received antibiotics (prophylactic kanamycin, 1 mg/kg), which were then allowed to recover from anesthesia.

Pulsed Radiofrequency Procedure

Following the establishment of mechanical and cold hypersensitivity on the ipsilateral hindpaw, rats with neuropathic pain behavior were randomly assigned to the PRF (+) and the PRF (−) groups (N = 6 for each group); in addition, the PRF procedure was performed on the ipsilateral DRG (right L5) at POD 10. In brief, under anesthesia, the right L5 DRG was exposed through laminectomy and facetectomy, without injury to the dura mater. PRF was then applied on the corresponding DRG, using a PRF machine (Cosman RFG-1A Generator; Radinics PFG 3C plus, Burlington, MA, USA), by placing the active tip electrode perpendicularly to the DRG. After carrying out the motor stimulation test, via muscle contraction of the lower extremities, PRF waves were then applied. The motor stimulation test was utilized instead of the sensory stimulation test. Stimulation parameters of PRF waves were set as follows: 2 bursts/s, duration = 20 milliseconds, output voltage = 45 V, maximum temperature = 42°C, and stimulated time = 180 seconds. All procedures in the PRF (−) group for control, including even placement of the electrode to the DRG, were identical to those in the PRF (+) group. However, the machine was turned off to not allow any RF stimulation to the DRG. Following the PRF procedure, rats were cared for using the same methods described earlier.

Pain Behavior

In order to ascertain the development of mechanical and cold hypersensitivity, rats were tested for mechanical and cold sensitivity of the plantar surface of the ipsilateral hindpaw, 1 day before, and 1, 4, 7, and 9 days after the operation. Behavioral tests were also performed throughout a period of 12 days after PRF by investigators blinded to the experimental group and protocol for each rat.

Mechanical Hypersensitivity

Mechanical hypersensitivity was tested by measuring the withdrawal response of the hindpaw to mechanical stimulation with the von Frey filaments (North Coast Medical Inc., Morgan Hill, CA, USA). The test procedures were described in the previous reports [17]. Briefly, the rats were placed in a clear plastic cage with a metal mesh floor, adapted to the testing environment for 15 minutes, and the plantar surface of each hindpaw was stimulated, in order to cause slight bending for 3–5 seconds with the von Frey filaments (0.16, 0.4, 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0, and 15.0 g) of increasing or decreasing thickness. It began with a 0.16 g probe, until a filament consistently gave withdrawal responses to more than three out of the five stimuli. Fifty percent probability thresholds of mechanical paw withdrawal were calculated. If no withdrawal response was elicited by the 15 g filament, 15 g was assigned as the mechanical threshold [18].
Cold hypersensitivity

Cold hypersensitivity was also determined by measuring the cold withdrawal response of the hindpaw to an acetone application. The rats were placed in a clear plastic cage with a metal mesh floor and adapted to the testing environment for 15 minutes before the measurements were taken; acetone was then applied to the lateral plantar surface of the ipsilateral hindpaw, corresponding to the L5 dermatome. An acetone drop was formed at the end of a small polyethylene tube, connected to a syringe and touched to the hindpaw. Each hindpaw was tested five times, only after the hindpaw was completely rested on the floor. A trial on each paw was repeated after a minimum interval of 5 minutes. Frequency of paw withdrawal was measured and the percent of the mean cold withdrawal was measured and the percent of the mean cold withdrawal frequency was calculated as follows: (no. of trials accompanied by brisk foot withdrawal) × 100/(no. of total trials) [19].

Immunohistochemical Staining and Quantitative Image Analysis

For immunohistochemical staining for microglial and astrocytic markers in the spinal cord, rats of the PRF (+) and the PRF (−) groups (N = 4 for each group) were sacrificed, at 12 days after PRF by perfusion fixation. Under anesthesia, a catheter was inserted into the left ventricle and was rinsed with 100 mL of saline, followed by 500 mL of 4% paraformaldehyde (in 0.1 N phosphate buffer [PB]). Spinal cords at the L3-S1 level were removed, post-fixed for 2 days in the same fixative, and were stored in 30% sucrose (in PB) for at least 24 hours. Transverse sections of the spinal cord of 30 μm thick were made using a cryostat (Leica, Wetzlar, Germany) and stored in PB. All incubation and reaction procedures for multiple immunohistochemical staining were performed at room temperature and on a shaker. To enhance the penetration of the antibody to the tissues, spinal cord sections were reacted with 50% ethanol for 30 minutes and rinsed with phosphate buffered saline (PBS) for 5 minutes, three times, and to block a nonspecific reaction of the secondary antibody, samples were treated with 10% normal donkey serum (NDS, Jackson ImmunoResearch, Westgrove, PA, USA). Tissue sections were incubated overnight in a mixture of the primary antibodies; mouse anti-OX42 (Cedarlane, Burlington, NC, USA; 1:200) and mouse anti-glial fibrillary acidic protein (GFAP) (Sigma, St. Louis, MO, USA; 1:500). Following the completion of a reaction with the primary antibody, tissues were rinsed with PBS for 5 minutes, three times, treated with 2% NDS for 10 minutes, and incubated with Alexa 488-conjugated goat anti-mouse (Invitrogen, Eugene, OR, USA; 1:200) for 3 hours, rinsed with PBS, and mounted with Vectashield (Vector Lab, Burlingame, CA, USA). All antibodies were tested for sensitivity and specificity before the study, and optimal dilutions were performed, according to the manufacturer’s recommendation. Immunofluorescent images were acquired with a cooled charge-coupled device (CCD) camera (F-View II, Soft Imaging System, Münster, Germany), which was attached to a light microscope (Leica DMR). For quantitative analysis of immunoreactivity for OX42 and GFAP in the dorsal horn, we obtained images from three spinal cord sections from L5 segments per rat of the PRF (+) and the PRF (−) groups. In each section, we acquired one image (898 × 660 μm) with a CCD camera, using the same shutter speed and digital gain. Images were encoded in order to blind the investigator before the analysis. Pixels positive for OX42 and GFAP immunoreactions were segmented by the application of an appropriate threshold of gray value and the area fractions (segmented area/total frame area) were calculated by an image analysis software (Analysis Pro, SIS, Münster, Germany).

Statistical Analysis

Collected data were encoded into SPSS/PC version 14.0 (SPSS Inc., Chicago, IL, USA) and analyzed. Data were reported as the mean ± standard deviation (SD). Statistical significance was set at P < 0.05. The baseline measures of behavioral tests were analyzed by a repeated measure one- or two-factor analysis for the within-group and between-group interaction. Quantitative image analysis for the glial immunoreactivities was performed using the Mann–Whitney U-test.

Results

In all experimental animals, pain behaviors of the ipsilateral hindpaw to the mechanical and cold stimuli were developed after the surgery of L5 SNL and transection. Changes in pain behavior were significant, compared with the basal value, and were maintained for 9 days in both groups. Following an application of PRF on the ipsilateral DRG after ascertainment of the development of neuropathic pain behavior, the withdrawal threshold to mechanical stimuli in the PRF (+) group was significantly reduced from 1 day after PRF, compared with that of the PRF (−) group (Figure 1A). They were maintained throughout a period of 12 days after a single application of PRF. However, between the two groups, there was no significant difference in the cold hypersensitivity, although the withdrawal frequency to cold stimuli was also reduced from day 1 after PRF (Figure 1B).

After an injury of the spinal nerve, immunoreactivity for OX42 and GFAP, microglial and astroglial markers, respectively, increased in the ipsilateral dorsal horn, compared with the contralateral dorsal horn in both groups. An increase of OX42-positive microglia in the PRF (+) group were attenuated, particularly in the lamina II and III, in the ipsilateral dorsal horn when compared with the PRF (−) group at 12 days after the PRF procedure (Figure 2A+B) (P < 0.05). Quantitative analysis of OX42 immunoreaction also revealed a significant reduction in the area fraction of OX42 immunoreaction of the dorsal horn in the PRF (+) group (average area fraction = 3.25%) when compared with that of the PRF (−) group (average area fraction = 7.1%) (Figure 2E) P < 0.05. In turn, although the immunoreactivity of GFAP in the ipsilateral dorsal horn was increased in both groups after a spinal nerve injury, the
Figure 1  The effects of pulsed radiofrequency (PRF) on mechanical allodynia (A) and cold allodynia (B) induced by L5 spinal nerve ligation and transection. After establishment of neuropathic pain (9 days after spinal nerve injury), PRF was applied for 180 seconds onto the dorsal root ganglion (DRG), corresponding to the injured spinal nerve at 10 days postoperatively. Statistically significant differences are indicated in the figures as follows: # From the basal value before spinal nerve injury; * From the value of the PRF (−) group after the PRF procedure ($P < 0.05$).

Figure 2  Immunoreactivities for OX42 in ipsilateral (A,C) and contralateral (B,D) spinal dorsal horn (L5) of the pulsed radiofrequency (PRF) (−) and the PRF (+) groups. At 12 days after PRF, the increase of the immunoreactivity for OX42-positive microglia was observed in the ipsilateral dorsal horn of the PRF (−) group and the increase was attenuated in the PRF (+) group. In quantitative analysis of the immunoreactivity, the area fraction of OX42-positive area was significantly reduced in the PRF (+) group, compared with the PRF (−) group (E) ($P < 0.05$). Values were expressed as mean ± standard deviation. Scale bar = 100 μm.
The expression of GFAP did not show any difference between the two groups (Figure 3A,C). Quantitative analysis of GFAP-positive area fractions after PRF also revealed no significant differences between the PRF (+) group (average area fraction = 15.18%) and the PRF (-) group (average area fraction = 12.21%) (Figure 3E).

**Discussion**

In our investigation, mechanical and cold hypersensitivity, induced by L5 SNL and transection, were alleviated after a single application of PRF on the ipsilateral DRG, and the activation of the microglia, which was significantly attenuated in the ipsilateral dorsal horn, was revealed in the PRF (+) group, compared with that of the PRF (-) group. In contrast to CRF, the PRF procedure has been known as a less destructive technique, with the absence of permanent neurological deficits, which has been applied as a treatment of various chronic pain conditions, with or without neuropathic pain. PRF has also been used for the treatment of patients with neuropathic pain after a peripheral nerve injury, and the neuropathic pain was reduced and maintained for a long time after a single application of the PRF procedure [10,11,20,21]. In an animal study, percutaneous application of PRF for 120 seconds reversed the mechanical allodynia, induced by an SNL [22]. In a neuropathic pain model, induced by an injection of the resiniferatoxin, which mimics the clinical features of postherpetic neuralgia, 5 weeks after PRF on the sciatic nerve significantly reduced the mechanical allodynia [23]. They also demonstrated that the PRF procedure was more effective when applied in an early stage (1 week PO), rather than in the late stages (5 weeks PO) of mechanical allodynia and with increased exposure time of PRF current. Furthermore, in a neuropathic pain model of rabbits, induced by partial sciatic nerve ligation, the Seltzer’s model [24] in rats, reduction of mechanical hyperalgesia and thermal hypersensitivity was observed as a result of PRF application to the corresponding DRG (L5 and L6) [25]. The present study further investigated the PRF effect, as an analgesic in a neuropathic pain model, induced by L5 SNL and transection, and showed a reduction of mechanical allodynia after PRF application on the DRG. Our findings were consistent with those of the previous studies.

On the 10 days PO, we arbitrarily applied PRF, when it is the early stage of neuropathic pain, and we could ascertain the maintenance of neuropathic pain, although pain developed at 1 day PO. We supposed that the evaluation of the effect of PRF for ongoing neuropathic pain conditions would be adequate. Optimal parameters for the PRF procedures are not known; according to the protocol described by Sluijter and Racz [26], the exposure time of PRF has been used at 120 seconds; however, in clinical practice, various exposure times have been chosen arbitrarily, from 60 seconds to 8 minutes [5,10,11,27,28]. Although it appears to be more effective when applied with increased exposure time of PRF current in animal studies [23], the implication of such exposure is not yet clear in its clinical practice. We applied PRF current at 42°C for 180 seconds, which appeared to be sufficient for the stimulation of the DRG. Other parameters of PRF, in this study, were also identical to those for clinical use in...
humans. We observed that pain behavior was not reversed to the basal value after PRF in this study. An association with another mechanism for the treatment of neuropathic pain could explain the incomplete resolution of neuropathic pain after PRF. Contribution of afferent input, from not only the injured nerve, but also the intact spinal nerves to the development of neuropathic pain, has been reported [29,30]. In clinical practice, simultaneous application of PRF on multiple levels of the adjacent dermatome is challenging in obtaining a better outcome; however, it remains unclear.

As such, the mode of action of PRF is not yet well understood. The biologic effect of PRF has been previously studied in normal animals. Early upregulation of the c-fos gene in the dorsal horn was induced after PRF, and suggested the PRF activation of pain-processing neurons in the dorsal horn via higher voltages and electromagnetic force, rather than tissue heating [14]. Late changes of c-fos immunoreactivities on the DRG were also reported after PRF [15]. Cellular stress, like the upregulation of the activating transcription factor 3 (ATF3) in small diameter neurons, was also observed after PRF on the L4 anterior primary ramus [13]. Furthermore, PRF could induce changes to the morphology of the cells. Electron microscopic findings of PRF on rabbit DRG showed changes in the cellular substructures, although normal DRG morphology was observed at the light microscopic level [31]. Ultrastructural changes in the axons after exposure of PRF, including abnormal membranes and morphology of mitochondria, as well as the disruption and disorganization of microfilaments and microtubules, were also reported. The degree of damage and disruption appeared to be greater in C- and Aδ-fibers than in Aβ-fibers [32].

Analgies effects of PRF in neuropathic pain model have also been reported [22,23,25]. However, only the behavioral changes after PRF application in a neuropathic pain state were observed, although they compared the difference in pain behavior, according to the following: variables of exposure time, duration of pain, and stimulation site of PRF. Moreover, little has been studied for the changes of glial expression in the spinal dorsal horn in association with pain reduction after PRF application in conditions with neuropathic pain. In this study, the reduction of the established mechanical hypersensitivity after PRF was observed, and significant attenuation of microglial expression in the spinal dorsal horn was observed in the PRF (+) group, compared with the PRF (−) group. Of particular interest, activation of the microglia, rather than astrocytes, in the dorsal horn was attenuated after an application of PRF on the DRG in this study. There have been reports suggesting that glial cells in the spinal dorsal horn play an important role in pain facilitation [33,34]. Peripheral nerve injury induced microglial activation and increased the expression of microglial markers in the spinal dorsal horn. Conversely, the microglial inhibitors can reduce the neuropathic pain after peripheral nerve injury [35]. Our results suggest that PRF on the DRG can alleviate the neuropathic pain, induced by a spinal nerve injury, similar to the previous studies, and the action of PRF for pain reduction may be associated with an attenuation of the spinal expression of microglia, rather than astrocytes. However, we could not explain how PRF application can inhibit the microglial expression in this limited study. Following a peripheral nerve injury, recruitment of the spinal microglia and their activation signaling pathway has been known for neuropathic pain transmission. Glial activation is induced by substances released from the primary afferent fibers in the dorsal horn, including the excitatory amino acids (EAAs), substance P, calcitonin-gene-related peptide (CGRP), nitric oxide (NO), prostaglandins (PGs), and ATP [30,33,34,36,37]. Conversely, activated glia release various pain-modulating substances, such as proinflammatory cytokines (TNF-α, IL-1, and IL-6), chemokines, PGs, EAAs, nerve growth factor (NGF), and reactive oxygen species [2]. According to the results of this study, it seems that PRF application on the DRG may modulate or interrupt the activities of primary afferent impulses from the injured neurons or neuron to microglial signaling in the spinal dorsal horn. However, the exact mechanisms are still unknown, and further studies are necessary for the determination of how PRF application modulates microglial expression and interactions between primary afferent neurons and microglia in the spinal dorsal horn. Moreover, the PRF procedure on non-nervous tissue, such as intra-articular, intradiscal, or transcutaneous application of PRF may even have an analgesic effect [38-40]. The possibility of another explainable mechanism for the analgesic effect of PRF can also be considered, and additional studies are necessary. In the future, the effect of PRF application on DRG in radiculopathic models, such as disc herniation, should be investigated. In addition, the optimal treatment time and frequency should be determined. The limitation of this study is that we could not investigate a sufficient duration after PRF and clarify the mechanism of interaction between PRF application on DRG and changes of the glial expression on the spinal dorsal horn. In conclusion, these results imply that application of PRF on DRG could mitigate neuropathic pain by the SNL and transection, and that the alleviation of neuropathic pain, after PRF, may be associated with the attenuation of microglial expression in the spinal dorsal horn.

Acknowledgments

This research was supported by a grant of Yeungnam University Medical Center (2008).

References


