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Pentoxifylline attenuates the development of hyperalgesia in a rat model of neuropathic pain

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Abstract

Pentoxifylline, a non-specific cytokine inhibitor, has shown to be beneficial in inflammatory pain in both experimental and clinical studies. The present study demonstrates for the first time, to our knowledge, the antihyperalgesic effect of pentoxifylline in the neuropathic pain using L5 spinal nerve transection rat model. In a preventive paradigm, pentoxifylline (12.5, 25, 50, or 100 mg/kg intraperitoneally) was administered systemically daily, beginning 1 h prior to nerve transection. Pentoxifylline (50, or 100 mg/kg i.p.) produced significant decrease in the mechanical and thermal hyperalgesia. However, pentoxifylline (100 mg/kg i.p.) did not influence the paw pressure thresholds and paw withdrawal latency in sham-operated rats. In order to understand the possible antinocicieptive effect of pentoxifylline in neuropathic pain, we examined the level of TNFα, IL-1β, IL-6 and IL-10 protein in the contralateral brain on day 7 post-transection. Pentoxifylline administration resulted in a dose-dependent reduction of the production of proinflammatory cytokines like TNFα, IL-1β and IL-6, and enhancement of IL-10. Furthermore, we investigated the activity of nuclear factor kappa B (NF-κB) in the contralateral brain on days 7 after surgery. In accordance with the change of proinflammatory cytokines, Pentoxifylline (50 or 100 mg/kg) significantly inhibited the activation of NF-κB in the brain. This research supports a growing body of literature emphasizing the importance of neuroinflammation and neuroimmune activation in the development of neuropathic pain states, and the potential preventive value of pentoxifylline in the treatment of neuropathic pain.

Keywords: Pentoxifylline; Neuropathic pain; Proinflammatory cytokines; IL-10; NF-κB

Neuropathic pain is initiated by a lesion or dysfunction in the nervous system. It is associated with severe, chronic sensory disturbances characterized by spontaneous pain, enhanced responsiveness to noxious stimulus (hyperalgesia), and pain in response to a normally nonnoxious stimulus (allodynia). Attempts to elucidate its mechanism have focused principally on peripheral nerves, dorsal root ganglion, and central nerve system (CNS) neurons. Recently, however, the neuroimmune activation and neuroinflammation, which are indicated by the microglia and astrocytes hypertrophy and proliferation and the production of proinflammatory cytokines in the CNS, have been considered to play a major role in the development and maintenance of neuropathic pain [4].

Mounting evidence has shown that cytokines, especially proinflammatory cytokines like tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), and IL-6, are strongly implicated in the genesis, persistence and severity of neuropathic pain. IL-10, which is known as a cytokines synthesis inhibiting factor, has also been found involvement in the pain [24]. The level of IL-10 was increases gradually in a rat chronic constriction injury (CCI) model [15], and its administration reduced the hyperalgesia induced by CCI injury [23]. A recent study has proved that intrathecal administration of a novel adeno-associated viral (AAV) 2-IL-10 vector encoding IL-10 was successful in transiently preventing and reversing neuropathic pain [14]. Nuclear factor kappa B (NF-κB), which is a key transcriptional factor in regulating the gene expression of proinflammatory cytokines, has been found activated in the pain. In animal models of neuropathic pain, activation of NF-κB has been detected in dorsal ganglia [13]. Furthermore, selective inhibition the activity of IκB kinase (which phosphorylates IκB to release NF-κB) attenuated

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the hyperalgesia in inflammatory and neuropathic pain models of rats [20].

Pentoxifylline, which is known as a non-specific cytokine inhibitor, has shown to be beneficial in clinical inflammatory related pain [26]. In rats of formalin-induced pain, the local injection of either pentoxifylline or propentofylline reduced the pain behavior, and the effect was associated with a decreased TNFα mRNA expression in the rat paws [8]. In a more comprehensive study about the antihyperalgesic effect of pentoxifyline on three experimental inflammatory pain models, the antinociceptive activity of pentoxifylline was proved to be associated with the inhibition of the release of both IL-1β and TNFα [21]. But until now, there has been little information about the use of pentoxifylline on experimental neuropathic pain models.

The objective of the present study was to determine whether preemptive systemic administration of pentoxifylline could attenuate the pain behavior in a rat model of neuropathic pain induced by L5 nerve transection, and whether the antihyperalgesic effect of pentoxifylline was associated with its ability to reduce the production of TNFα, IL-1β, and IL-6 through the inhibition of NF-κB, and to stimulate the expression of IL-10 in the brain.

Male Sprague-Dawley rats weighing 190–220 g at the start of surgery were used. The animals were housed on a 12-h light, 12-h dark cycle with food and water available ad libitum. In accordance with the guidelines set forth by the National Institutions of Health regarding the care and the use of animals for experimental procedures, efforts were made throughout to minimize animal discomfort and to use the fewest animals needed for statistical significance.

Neuropathic pain was induced using the methods described by Kim and Chung [12]. Animals were deeply anesthetized with halothane in an O2 carrier (induction, 4% and maintenance, 2%). A small incision to the skin overlaying L5-S1 was made, followed by retraction of the paravertebral musculature from the vertebral transverse processes. The L6 transverse process was partially removed, exposing the L4 and L5 spinal nerves. The L5 spinal nerve was identified, lifted slightly, ligated tightly with a 3-0 silk thread and transected. The wound was irrigated with saline and closed in two layers with 3-0 silk threads. In a systemic preventive paradigm 12.5, 25, 50, or 100 mg/kg pentoxifylline (Sigma–Aldrich, St. Louis, MO, USA) or saline vehicle ($n = 8$/treatment) was given by the intraperitoneally route 1 h before surgery and continued daily to day 7 post-transection. Besides two groups of animals treated with 100 mg/kg pentoxifylline or saline vehicle ($n = 8$/group) in the above-mentioned scheme were utilized to receive sham surgery to identify the possible direct analgesic effect of pentoxifylline. All injections were completed 16 h prior to the behavioral testing. The doses were selected based on the previous studies about pentoxifylline on inflammatory pain and its analog-propentofylline on formalin-induced pain, neuropathic pain [8,18,21,26]. The experimenter was blinded to drug treatment.

Behavioral studies were carried out in a quiet temperature-controlled (24 °C) room between the hours of 8:00 AM and 10:00 AM. All the behavior was recorded before surgery, and on post-nerve transection days 1, 4, 7. Mechanical nociceptive thresholds were measured by using an Electro VonFrey anesthesiometer (Model 2390CE, IITC Life Science, INC.). Rats were placed singly beneath an inverted ventilated Plexiglas cage with a metal-mesh floor allowing access to the plantar surface of hind paw. After 10 min for acclimation, gentle incremental pressure (maximum 200 g) by a rigid von Frey hair was applied to the dorsal surface of the ipsilateral hind paw until the paw was withdrawn. Five tests were conducted at intervals of 5 min and the force (g) applied was recorded.

Thermal sensitivity was determined by using paw withdrawal latencies to radiant heat (Model 390, IITC Life Science, INC.). After animals were acclimated to the Plexiglas cage with a 6-mm thick glass floor, the radiant heat source beneath the glass floor was focused on the plantar surface of the ipsilateral hind paw when in contact with the floor. The paw withdrawal latencies were obtained per animal for five times with intervals of 5 min. Light intensity was preset to obtain a baseline latency of approximately 10 s and the cutoff time was set at 20 s to avoid the tissue damage.

After the behavioral testing on day 7 postsurgery, all rats were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and rapidly decapitated ($n = 8$/group). The brain was removed quickly to liquid nitrogen for assays.

Quantitative determination of TNFα, IL-1β, IL-6 and IL-10 protein was performed on the brain tissue harvested on day 7 postsurgery. The anterior half brain tissue (mainly including prefrontal cortex, weighing 150–200 mg), excluding the olfactory blubs, which was contralateral to the operation side, was dissected [22], and homogenized in homogenization buffer consisting of a protease inhibitor (Roche Diagnostics, Mannheim, Germany) using Power Gen 124 tissue tearer (Fisher Scientific Co., Suwanee, GA). Samples were spun at 20,000 × g for 30 min at 4 °C. Supernatant was aliquoted and stored at −80 °C for future protein quantification. TNFα (Diaclone Research, Besançon, France), TL-1β, IL-6 and IL-10 (Biosource International, Camarillo, CA, USA) were determined using the quantitative sandwich enzyme immunoassay according to the manufacturer's protocol. Values were expressed as pictogram per milligram protein.

Electrophoretic mobility shift assay (EMSA) for NF-κB was performed using a commercial kit (Gel Shift Assay System, Promega, Madison, WI, USA). The method has been described [27]. Briefly, equal amounts of nuclear extract (10 μg) were added to 9 μl of gel shift binding buffer for 15 min at room temperature. Then the mixture was incubated for 30 min with 1 μl of ^{32}P-labelled oligonucleotide probe. Last 1 μl of loading buffer was added and the sample was electrophoresed in a 4% polyacrylamide gel. The dried gel was exposed to X-ray film (Fuji Hyperfilm) at −70 °C for 48 h. The intensity of the NF-κB was assessed by the densitometry.

Values were expressed as means ± S.D. Comparison between groups were performed by two-way analysis of variance (ANOVA) for repeated measurements followed by Tukey's test (SPSS 12.0). $p < 0.05$ was considered significant.

Before L5 spinal nerve transection, all groups exhibited comparable baseline thresholds to the noxious mechanical or thermal stimuli. L5 spinal nerve transection produced marked mechani-

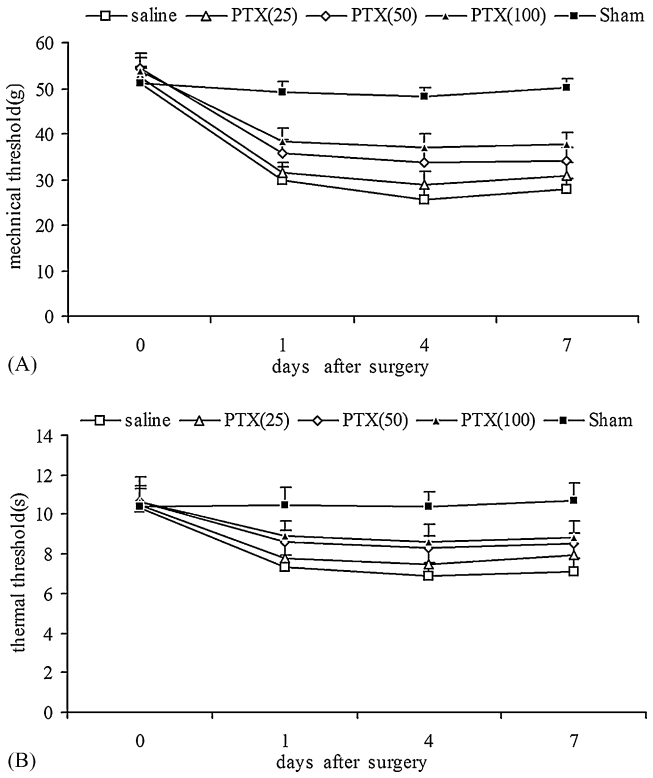

Fig. 1. Changes in the paw withdrawal, (A) mechanical threshold and (B) thermal latency, on day before surgery and days 1, 4, 7 after operation. Nerve injury resulted in an overall statistically significant ($p < 0.01$) decrease in mechanical and thermal threshold compared with animals in the sham-operated group. Pentoxifylline (100 or 50 mg/kg i.p.) treatment attenuated the development of mechanical and thermal hyperalgesia in L5 nerve-transected rats compared with the saline treated nerve-transected rats ($p < 0.01$ for 100 mg/kg and $p < 0.05$ for 50 mg/kg).

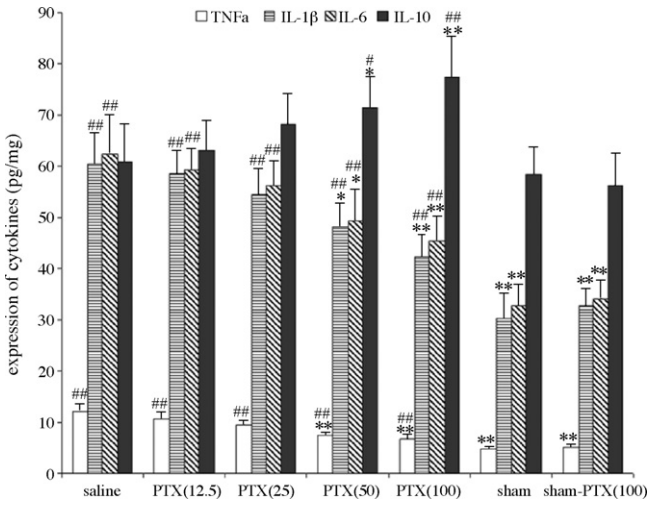

Fig. 2. Expression of the TNFα, IL-1β, IL-6 and IL-10 in the brain on days 7 post-transection. TNFα, IL-1β, and IL-6 production was markedly elevated in nerve injury rats compared with the sham-operated animals, pentoxifylline (100 or 50 mg/kg i.p.) treatment significantly reduced the expression TNFα, IL-1β, and IL-6, but significantly enhanced the excretion of IL-10. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. saline group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. sham group.

cal and thermal hyperalgesia, indicating by mean paw pressure thresholds and paw withdrawal latency significantly decreased compared with animals in the sham-operated saline treated group (Fig. 1). Pentoxifylline (50 or 100 mg/kg i.p.) administration initiated 1 h before surgery attenuated the development of mechanical and thermal hyperalgesia in L5 nerve-transected rats compared with the saline treated nerve-transected rats ($p < 0.01$ for 100 mg/kg and $p < 0.05$ for 50 mg/kg). Whereas low dose pentoxifylline (25 or 12.5 mg/kg i.p.) did not show any significant difference in the mechanical and thermal hyperalgesia ($p > 0.5$) compared with saline treated nerve-injuried rats (data not shown for pentoxifylline 12.5 mg/kg group). Furthermore, the behavioral values of sham-operated rats were steady during the experimental period and no difference was found between those treated by pentoxifylline (100 mg/kg i.p.) and saline (data not shown for sham-pentoxifylline group).

Attenuation of behavioral mechanical and thermal hyperalgesia by pentoxifylline after nerve injury could be due to its inhibitory effect on the production of inflammatory cytokines, so we studied the level of inflammatory cytokines and anti-inflammatory cytokine "IL-10" in the brain on day 7 after operation (Fig. 2). TNFα, IL-1β, and IL-6 levels in the brain were significantly increased in nerve-injuried rats compared with the sham-operated saline treated rats ($p < 0.01$ for all surgery groups). Pentoxifylline (50 or 100 mg/kg i.p.) administration significantly reduced the production of the TNFα, IL-1β, and IL-6 in the brain compared with the saline treated nerve-transected rats ($p < 0.01$ for three cytokines of 100 mg/kg and TNFα of 50 mg/kg, and $p < 0.05$ for IL-1β and IL-6 of 50 mg/kg group). On the contrary, the low dose pentoxifylline (25 or 12.5 mg/kg i.p.) had no significant effect on the expression of TNFα, IL-1β, and IL-6 in the brain compared with the saline treated nerve-transected rats ($p > 0.5$). Just like the proinflammatory cytokines, nerve transection also enhanced the excretion of IL-10 in the brain, but in contrast to the TNFα, IL-1β, and IL-6, IL-10 was significantly increased in the pentoxifylline (50 or 100 mg/kg i.p.) treated nerve-transected rats compared with those treated with the saline ($p < 0.01$ for 100 mg/kg and $p < 0.05$ for 50 mg/kg). There was no significant difference between pentoxifylline (12.5 or 25 mg/kg i.p.) treated nerve-transected rats and those with saline. Coincidence with the behavioral data, the production of TNFα, IL-1β, IL-6 and IL-10 in the brain was similar between sham-operated rats administered with pentoxifylline (100 mg/kg i.p.) or saline ($p > 0.5$).

EMSA experiments were undertaken to examine the effect of pentoxifylline on the activation of NF-κB induced by L5 nerve transection. As shown in Fig. 3, NF-κB activity was significantly elevated in the nerve injury rats compared with rats received sham surgery ($p < 0.01$ for all groups). Pentoxifylline (50 or 100 mg/kg i.p.) significantly inhibited the activation of NF-κB compared to saline in the nerve-transected rats ($p < 0.01$ for both).

This study examined the potential preventive value of pentoxifylline in the treatment of neuropathic pain using a L5 spinal nerve transection rat model of neuropathic pain. Our data showed that pentoxifylline dose-dependently attenuated mechanical and thermal hyperalgesia after nerve injury in a preventive administration paradigm. Howerever, the phe-

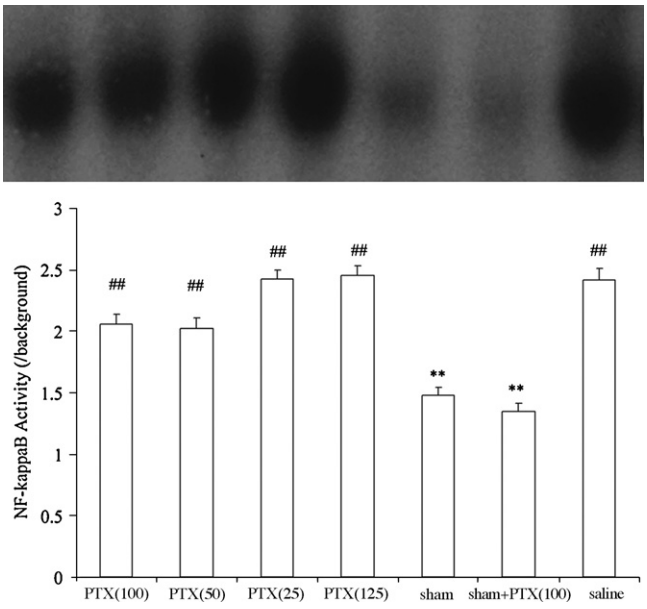

Fig. 3. Activity of nuclear factor kappa B (NF-κB) in the brain on days 7 after surgery. NF-κB activity was very low in the sham-operated saline-treated and sham-operated pentoxifylline (100 mg/kg i.p.) treated groups (column 5, 6). It was markedly increased in the nerve-injuried groups (column 1–4, 7). Pentoxifylline (100 or 50 mg/kg i.p.) treatment significantly inhibited the activation of NF-κB compared with saline group. $^{*}p<0.05$, $^{**}p<0.01$ vs. saline group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. sham group.

nomenon that pentoxifylline (100 mg/kg i.p.) administration does not influence the nociceptive threshold of mechanical and thermal stimuli in the sham-operated rats indicated that pentoxifylline has no specific direct anlagesic effect. The antihyperalgesic effect of pentoxifylline should be related to a reduced inflammatory response as what it played in inflammatory pain [8,21,26].

It is well established that central neuroimmune activation and neuroinflammation mediate and/or modulate the pathogenesis of neuropathic pain [6]. In the spinal cord, it is believed that nerve injury leads to the release of the microglial stressors, such as adenosine triphosphate (ATP), nitric oxide (NO), substance P, excitatory amino acid, and Toll-like receptor 4 (TLR4) of microglial plays a crucial part as a receptor in the activation of microglial in rodent models of neuropathy [4,19]. The activated glial cells release a host of proinflammatory cytokines, such as TNFα, IL-1β, and IL-6, which induce a long-term alteration of synaptic transmission in the CNS and play a critical role in the development and maintenance of neuropathic pain [6]. In both the peripheral and central models of neuropathic pain, the elevated levels of IL-1β mRNA and protein are associated with hyperalgesia and allodynia in response to injury [9,25]. Using both in situ hybridization and RNase protection assay (RPA), IL-6 mRNA is increased in response to peripheral and nerve root injury [1]. Moreover, intrathecal injections of an IL-6 neutralizing antibody significantly reduced nerve injury-induced mechanical allodynia [2]. Similar to IL-1β and IL-6, the level of TNFα mRNA and protein is increased during neuropathic pain [3,10]. Inhibition of proinflammatory cytokines using a cocktail of IL-1β and TNFα antagonists attenuates pain-associated behavior in a dose-dependent manner in rats with neuropathic pain [17].

In the present study, we investigated the level of TNFα, IL-1β, and IL-6 in the brain instead of the lumbar spinal cord on day 7 after nerve transection. As previous studies reported that the level of proinflammatory cytokines and the mRNA of IL-1β and TNFα increased in the brain in the rats models of neuropathic pain [3,22,27], we also found that nerve injury resulted in the elevated production of TNFα, IL-1β, and IL-6 in the contralateral brain. Additionally, the antinociceptive effect of pentoxifylline was in accordance with the reduced level of those cytokines in the brain induced by pentoxifylline. Those results indicated that systemic administration of pentoxifylline would inhibit the activation of glial cells, which are the primary sources releasing inflammatory cytokines, after spinal nerve transection in the brain and result in the antihyperalgesic effect. Compared with spinal cord, it seemed that peripheral nerve injury was too far to send any direct signals to activate the glial cells in the brain. But the sensitization and activation of ascending spinal dorsal neurons would release more excitatory transmitters in the contralateral supraspinal brain region, such as, substance P and excitatory amino acids, which might directly activate the supraspinal glial cells or induce neuronal depolarization combined with ion changed that may be major stimuli for glial cells activation [5].

In addition, we found that pentoxifylline dose-dependently stimulate the production of IL-10 in the brain after nerve injury, which in turn may suppress the production and activity of proinflammatory cytokines at several levels, including transcription, translation and release [7]. This enhancement effect may also participate in the antihyperalgesic effect of pentoxifylline after nerve injury.

In order to better understand the effect of pentoxifylline on cytokines, we measured the activity of NF-κB in the brain which is a key transcriptional factor in regulating of inflammatory cytokines. Our data showed that pentoxifylline dose-dependently inhibited the activation of NF-κB in the brain, but the effect was rather “all or not” instead of linear with the dose of pentoxifylline. Furthermore, the fact that the level of proinflammatory cytokines was intimately connected with the activity of NF-κB strongly indicated that pentoxifylline inhibited the production of proinflammatory cytokines through the NF-κB. With regard to the effect of pentoxifylline on cytokines, a previous study has demonstrated that pentoxifylline regulates the transcription of a particular cytokine through inhibition of NF-κB and nuclear factor of activated T cells (NF-AT), and stimulation of activator protein-1 (AP-1) and cAMP response element binding proteins (CREB) in human T cells [11]. Furthermore, the effect of pentoxifylline upon cytokines production seems to be due, at least in part, to an increase in intracellular cAMP levels in inflammatory cells [16].

In summary, we have demonstrated that preventive systemic administration of pentoxifylline would dose-dependently attenuate the mechanical and thermal hyperalgesia in L5 spinal nerve transection rats. The antinociceptive effect of pentoxifylline would be correlated with the reduction of the production of TNFα, IL-1β, and IL-6 through inhibiton of NF-κB, and the

stimulation the expression of IL-10 in the brain. However, the therapeutic effect of pentoxifylline on establishing neuropathic pain remains to be determined in the future study.

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