Journal of Pain Research

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ORIGINAL RESEARCH

The Antiallodynic Effect of Nefopam on Vincristine-Induced Neuropathy in Mice

This article was published in the following Dove Press journal:
Journal of Pain Research

Jin Young Lee[1]
Woo Seog Sim[1]
Noo Ree Cho[2]
Bae Wook Kim[2]
Jeong Yeon Moon[2]
Hue Jung Park[2]

[1]Department of Anesthesiology and Pain Medicine, Samsung Medical Center, Sungkyunkwan University, School of Medicine, Seoul, Korea; [2]Department of Anesthesiology and Pain Medicine, Seoul St. Mary's Hospital, The Catholic University of Korea, College of Medicine, Seoul, Korea

Background: Chemotherapy-induced neuropathic pain is a disabling condition following cancer treatment. Vincristine has more neurotoxicity than other vinca alkaloid agents. This study evaluated the correlation of different doses of nefopam with antiallodynic effects in a mouse vincristine neuropathy model.

Methods: A peripheral neuropathic mouse model was made by intraperitoneal injection of vincristine (0.1 mg/kg/day; 5-day-on, 2-day-off schedule over 12 days). After the development of allodynia, mice were injected intraperitoneally with 0.9% normal saline (NS group) or various doses (10, 30, 60 mg/kg) of nefopam (Nefopam group). We examined allodynia using von Frey hairs pre-administration and at 30, 60, 90, 120, 180, 240 mins, and 24 hrs after drug administration. We also measured the neurokinin-1 receptor concentrations in the spinal cord to confirm the antiallodynic effect of nefopam after drug administration.

Results: The peripheral neuropathic mouse model showed prominent mechanical allodynia. Intraperitoneal nefopam produced a clear dose-dependent increase in paw withdrawal threshold compared with pre-administration values and versus the NS group. The concentration of neurokinin-1 receptor was significantly decreased in the Nefopam group ($P<0.05$).

Conclusion: Intraperitoneally administered nefopam yielded a dose-dependent attenuation of mechanical allodynia and decreased neurokinin-1 receptor concentration, suggesting that the neurokinin-1 receptor is involved in the antiallodynic effects of nefopam in vincristine neuropathy.

Keywords: allodynia, mice, nefopam, neurokinin-1, neuropathy, vincristine

Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is a disabling condition following cancer chemotherapy, characterised by sensory (hyperalgesia, paresthesia, dysesthesia, allodynia, spontaneous pain), motor (weakness, incoordination), and autonomic (altered thermoregulation, blood pressure, intestinal motility) symptoms.[1,2] Although the pathophysiology of CIPN is not clear, it probably results from loss of sensory terminals in the skin; alterations of membrane receptors; and changes in intracellular signaling, neurotransmission, excitability, and cellular metabolism.[3] A reduction in Meissner's corpuscles and loss of epidermal nerve fibers are other possible mechanisms of CIPN.[4] The development of CIPN may lead to a reduction in dose or discontinuation of chemotherapy, which can result in increased cancer morbidity and/or mortality.[1,3] Because of these multiple mechanisms for the development of CIPN, so far, no agent has been reported to be successful in controlling CIPN optimally. Vincristine is one of the most commonly used chemotherapeutic agents for blood cancer and solid tumors.[5,6] Vincristine has

Correspondence: Hue Jung Park
Department of Anesthesiology and Pain Medicine, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul 06591, Korea
Tel +82 2-2258-6157
Fax +82 2-537-1951
Email huejung@catholic.ac.kr

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DovePress
http://doi.org/10.2147/JPR.S224478

been known to induce microtubule destabilisation and deficits in axonal transport, and thus, has more neurotoxicity than other vinca alkaloid agents.[7,8]

Nefopam has drawn interest for its antinociceptive and antihyperalgesic effects; it acts by influencing monoamines, and the noradrenergic and/or serotonergic system, resulting in the prevention and reduction of central neural sensitisation.[9–11] In a study with spinal nerve ligation models, intrathecal nefopam produced an antiallodynic effect by inhibition of microglial and astrocytic activation, which are reportedly involved in the release of nociceptive mediators and facilitation of the pain process pathway.[12] However, the effect of nefopam on allodynia in vincristine neuropathy and the precise mechanism involved are not well understood. Gautam et al described that the neurokinin-1 (NK1) receptor is functionally associated with mechanosensitive nociceptors and neurotransmission at the spinal level.[13] They reported that intrathecal administration of NK1 antagonist resulted in an antiallodynic effect in the post-incisional pain model.[13] The NK1 receptor antagonist inhibits excitatory postsynaptic potential in the dorsal horn neurons, resulting in attenuation of spinothalamic neuron activation and prevention of nociceptive sensitisation.[13,14] Therefore, we evaluated the correlation of different doses of nefopam with the antiallodynic effect in the mouse vincristine neuropathy model. In addition, the concentration of NK1 receptor in the spinal cord was assayed.

Materials and Methods

This study was reviewed and approved by the Animal Care and Use Committee of Seoul St. Mary's hospital of the Catholic University of Korea. The animals were treated in accordance with the National Institute of Health and the International Association for the Study of Pain policies.

Experimental Animals

Adult male mice (age, 10 weeks old; weight, 25–30 g) were used in this study. All animal experiments were performed in a semi-pathogen-free barrier zone at the Catholic Laboratory Animal Research Center. The mice were housed 2 or 3 per cage for 7 days in humidity and temperature-controlled (21–23°C) environment, with a 12 hr light/dark cycle (7:00 am hour onset) and free access to food and water at all times. Behavioural testing and analgesiometry were performed according to the ethical guidelines, and the mice were euthanised after completion of the planned tests.

Vincristine Injection

The vincristine-induced peripheral neuropathy model was used in this experiment. Baseline response to mechanical stimulation of the hind paw was established on day 1. Either vincristine sulfate (Sigma Aldrich Co., St. Louis, MO, USA) or saline was administered by injection to create the neuropathy model, as described previously.[15] In brief, 0.1 mg/kg/day vincristine was administered intraperitoneally for 5 days. Following cessation for 2 days, injection was continued for the next 5 days. On day 15, the paw withdrawal threshold (PWT) was measured with von Frey filaments (Semmes-Weinstein monofilaments, Stoelting Co., Wood Dale, IL, USA). If a foot withdrawal response occurred when a filament less than 0.6 g was applied to the hind paw, it was considered that mechanical allodynia had developed.

Drug Administration

Mice that showed mechanical allodynia were randomly divided into four groups before drug administration. The control group received distilled water 1 mL/kg (n=6). There were 3 experimental groups, namely, nefopam10, 30, and 60 (n=6, in each group), who were administered 10, 30, or 60 mg/kg nefopam (Acupan®, Pharmbio Korea, Seoul, Korea), respectively, intraperitoneally.

Behavioural Tests

All behavioural tests were conducted at fixed times (1:00–6:00 pm) to avoid circadian rhythm errors by the same examiner who was blinded to drug administration and dosing. After intraperitoneal drug administration, mice were placed on a metal mesh covered with a plastic dome (8 - 8 - 8 cm) for the assessment of mechanical allodynia. The PWT in response to mechanical stimulation was measured using the up-and-down method,[16] applying a series of von Frey filaments ranging from 0.03 to 2.00 g. Brisk withdrawal and paw flinching were considered positive responses. If the mice showed a positive response, the next-less-stiff filament was used for the next trial, or the next stiffer one in cases where there was no withdrawal or licking. Mice that did not show allodynia (PWT less than 0.6 g) were excluded from the study. PWT was assessed before drug administration, and at 30, 60, 120, 180, 240 mins, and 24 hrs after drug administration.

Immunohistochemistry

We measured the NK1 receptor in the spinal cord to confirm the antiallodynic effect of nefopam. All the mice

in each of the groups were sacrificed, and the spinal cords (lumbar 4–6) were collected 30 min after nefopam and vehicle injection (n=6, in each group), according to the mechanical allodynia results. Mice injected with saline and mice treated with 60 mg/kg nefopam were anaesthetised and perfused transcardially with 50 mL of 4% paraformaldehyde dissolved in 0.01 M phosphate-buffered saline (PBS) with ph 7.2–7.4. The spinal cords of the mice were then dissected. All obtained tissues were postfixed, and immersed in a 30% sucrose solution overnight. Spinal cord segments were cut into 10 µm thick slices on a freezing microtome. The slices were incubated with a rabbit polyclonal antibody against the NK1 substance P (SP) receptor (1:1000; catalog no. AB5060; Chemicon, Temecula, CA, USA) overnight at 4°C.[17,18] After the sections were washed with buffer, they were exposed to the secondary antibody, an anti-rabbit IgG antibody conjugated with Alexa-488 (1:500; Invitrogen, Carlsbad, CA, USA). The stained sections were cover-slipped and examined using a Zeiss LSM 510 Meta confocal microscope (Zeiss, Oberkochen, Germany), and the mean intensity was measured using Image-Pro Plus v.6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical Analysis

All data were analysed using GraphPad Prism (version 5.0; GraphPad Software, Inc., San Diego, CA). Data are expressed as the mean ± standard error of mean (SEM). Time-response data are presented as PWT to mechanical stimulation. Variables measured at different time points were compared using the repeated-measures analysis of variance test, with the Bonferroni post hoc correction, performed when appropriate. A *P*-value of less than 0.05 was considered to indicate statistical significance.

Results

Effect of Vincristine Injection on the Nociceptive Threshold

None of the vincristine-injected mice showed any motor dysfunction or complications. The vincristine-injected mice did not gain weight during the injection schedule (data not shown). However, they looked healthy. Saline did not induce any hyperalgesia or allodynia. Vincristine injection produced significant mechanical allodynia after the 12-day injection schedule ($P<0.0001$) (Figure 1). PWT was significantly reduced after vincristine treatment from day 5 to 15 compared with the baseline (Figure 1).

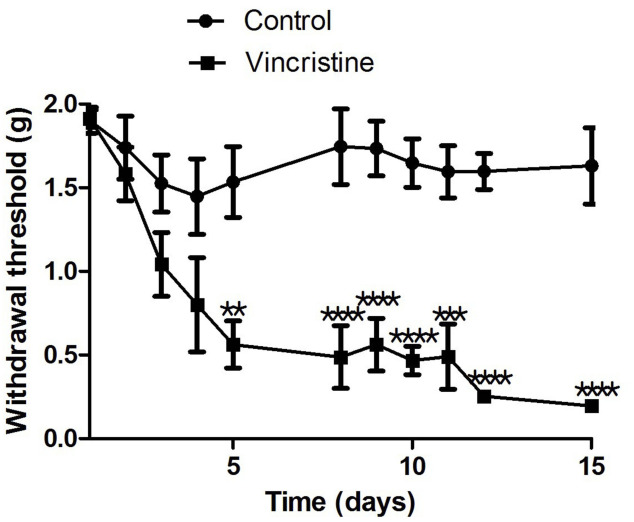

Figure 1 Time course of the paw withdrawal response to mechanical stimuli during vincristine treatment. The results are expressed as mean ± standard error of mean (n=6, in each group). ***P*<0.01, ****P*<0.001, *****P*<0.0001 significantly different from the saline group.

Mechanical Allodynia

The effects of nefopam on mechanical allodynia are shown in Figure 2. Intraperitoneal nefopam produced a clear dose-dependent increase in the PWT compared with the NS group. There was no significant change in the PWT in the NS group at any time point after injection. The greater dose groups (nefopam30 and 60) showed a more sustained increase in PWT against mechanical stimuli compared with the nefopam10 group. The antiallodynic effect of nefopam was

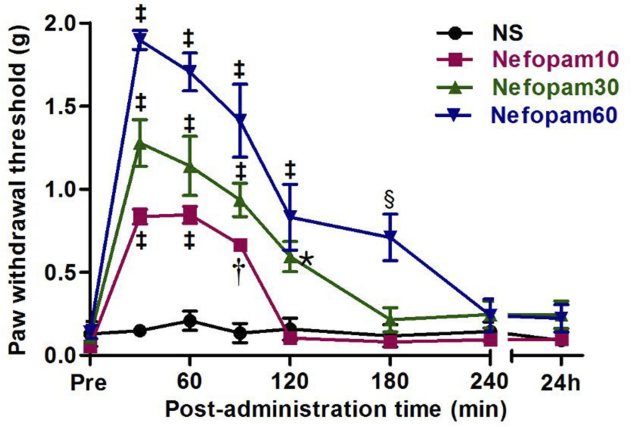

Figure 2 Effects of intraperitoneal nefopam on mechanical stimuli. The paw withdrawal threshold was measured before (Pre) and after intraperitoneal administration of normal saline (NS) and nefopam 10 mg/kg (Nefopam10), nefopam 30 mg/kg (Nefopam30), and nefopam 60 mg/kg (Nefopam60). The results are expressed as mean ± standard error of mean (n=6 in each group). ‡*P*<0.05 between the NS group and other groups, †*P*<0.05 between the NS and nefopam10 group, **P*<0.05 between nefopam30 and the NS, nefopam10 group, §*P*<0.05 nefopam60 vs other groups.

maintained until 90 mins in nefopam10, 120 mins in nefopam30, and 180 mins in the nefopam60 group (P<0.0001).

Neurokinin-1 Receptor

Immunohistochemistry showed that the NK1 receptor components increased in vincristine-induced neuropathic mice (P=0.004; Representative images in Figure 3B) than in a control mouse (Figure 3A). The increased immunoreactivity of the NK1 receptors was abolished after nefopam treatment in the vincristine-induced neuropathic spinal cord (P<0.01) (Figure 3C). The percentage of NK1 receptors was lower in mice that received nefopam treatment (P<0.05) (Figure 3D). The NK1 receptor concentration was 36.4±4.2 before vincristine injection, 80.6±7.9 after vincristine injection, and 45.6±4.7 after nefopam injection.

Discussion

In the present study, intraperitoneal nefopam produced a dose-dependent antiallodynic effect and attenuation of NK1 receptors in the dorsal root ganglia in vincristine-induced neuropathy mouse model. Our results suggest that nefopam promotes anti-nociception via the reduction of NK1 receptor, subsequently resulting in the inhibition of substance P (SP) and NK1 receptor signaling. However, we did not measure the circulating SP levels or NK1 receptor internalization; therefore, an additional study will be needed to investigate the change of NK1 receptor internalization and SP levels in mice with blunted NK1 receptor.

Cancer chemotherapeutic agents, such as vincristine, paclitaxel, and cisplatin are known to exert effects on the

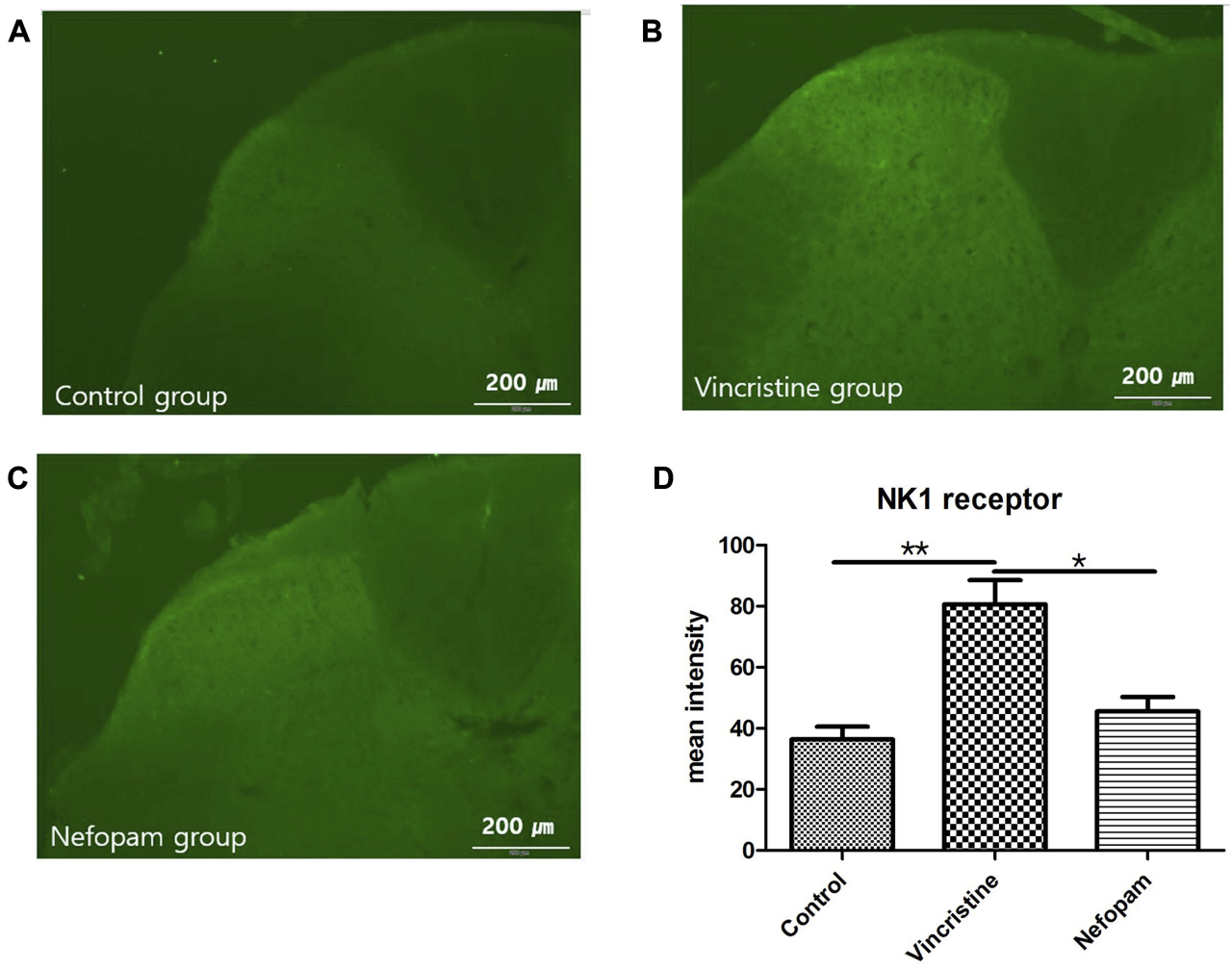

Figure 3 Effects of intraperitoneal nefopam on NK1 receptors after vincristine-induced neuropathy. Immunohistochemistry showed that NK1 receptor binding components were elevated in vincristine-induced neuropathic mice. (**A**) Representative spinal cord stained for NK1 receptor in a control mouse. (**B**) Representative spinal cord stained for NK1 receptor in a vincristine-induced neuropathic mouse. (**C**) Representative spinal cord stained for NK1 receptor in a vincristine-induced neuropathic mouse after 60 mg/kg nefopam treatment. (**D**) The percentage of NK1 receptors in the spinal cord was significantly lower in 60 mg/kg nefopam-injected mice (Nefopam) compared to saline-injected mice (Vincristine) in vincristine-induced neuropathic mouse models. *P<0.05, **P<0.01.

sensory nerve by altering the amplitude of the action potential, and conduction velocity, thereby, inducing neuropathic pain.[4,19] In this pathophysiology, voltage gated-sodium, calcium, and potassium channels, glutamate activated N-methyl-D-aspartate (NMDA) receptors, which play a significant role in the nociceptive process,[20] and increased SP release in the spinal cord are associated with cytotoxicity to the axons and neuronal cell bodies.[19,21] The incidence of CIPN varies from 3% to 38% with combination protocols. It can occur as early as during the first treatment and may last indefinitely. If the painful neuropathy persists for more than 3 months after therapy has ended, it is regarded as refractory to improvement.[4] Various drugs including anticonvulsants, tricyclic antidepressants, alpha-adrenergic agonists, ginkgo biloba extract, and opioids have been introduced to treat CIPN; however, the cause, persistence, optimal analgesics, and prevention regimes have not been fully documented.[22–24] Long-term therapeutic effects have been reported with opioids,[4] but the risks of tolerance, hyperalgesia, dependence, addiction, and immune suppression should be considered. Moreover, sensitivity to opioid analgesics may be reduced by the loss of opioid receptors on C-fibers, activation of NMDA receptors, or increased release of excitatory amino acids in some neuropathic pain states.[22]

Nefopam is a non-opioid, non-steroidal benzoxazocine-derived analgesic that inhibits monoamine reuptake in the central nervous system.[25,26] It indirectly modulates the NMDA receptor, inhibiting c-Fos expression in the dorsal horn of the spinal cord and relieving allodynia in rats.[11] Nefopam was first introduced as an antidepressant and anticonvulsant and was then reported to be efficacious in postsurgical hyperalgesia.[27] Several studies have demonstrated the anti-nociceptive effects of nefopam using a variety of routes of administration and for various indications.[9–11,28–30] Nefopam reduced thermal hypersensitivity after acute and postoperative pain in rats.[31] Intraperitoneal nefopam showed an antiallodynic effect mediated by adenosine triphosphate-sensitive potassium channel in rats with spinal nerve ligation-induced neuropathy.[9] The study suggested that the prolonged preemptive effect of nefopam is a result of inhibition of central neuroplasticity mechanisms after nerve injury.[9] Dam et al reported that intrathecal nefopam produced an antiallodynic effect by acting on the serotonin system (5-hydroxytryptamine) in neuropathic rats.[29] However, the precise mechanism of action of nefopam in vincristine-induced neuropathy is not clear.

In our study, we used nefopam in doses starting from 10 mg/kg, and it showed an antiallodynic effect. Measuring the concentration of NK1 receptors in postsynaptic dorsal horn neurons has been used to indicate the release of SP into the spinal cord.[32] SP is the main excitatory neurotransmitter of afferent C-fibers and contributes to the development and maintenance of neuropathic pain.[33,34] SP is an important molecule in the neurogenic inflammation pathway and plays a key role in eliciting pain sensation in the central and peripheral nervous system.[35] SP release increases in the spinal dorsal horn following the stimulation of peripheral nociception.[36] Elevated SP levels have been reported in the cerebrospinal fluid of patients with fibromyalgia and a chronic pain disorder.[37,38] The biologic actions of SP can be mediated through activation of the NK1 receptor, which is known to have a higher affinity for SP than the NK2 and NK3 receptors.[39] The released SP can mediate its effect on the NK1 receptor via two different effector systems, and promote neuronal hyperpolarization, which is associated with inflammatory allodynia and hyperalgesia.[35,39] Several studies have attempted to clarify the mechanism underlying the SP mediated responses and NK1 receptor internalization to investigate the feasibility of using NK1 receptor antagonist as a potential analgesics.[35,40,41] Our study demonstrated the analgesic effect of nefopam by validating NK1 receptor attenuation, which may reduce SP and NK1 signaling and lead to decreased nociceptive response.

Limitations

This study had some limitations. We did not examine cold allodynia. Also, we did not evaluate other inflammatory chemical mediators including pro-inflammatory cytokines (tumor necrosis factor α, interleukin (IL)-1, IL-6, and interferon α) and chemokines related with CIPN.[42] Further studies are needed to investigate the effectiveness of vincristine-induced neuropathy in cancer patients and related chemical mediators.

Conclusion

This study is the first to investigate the effects of intraperitoneal nefopam on allodynia in vincristine-induced neuropathy. The increased concentration of NK1 receptors as a result of vincristine administration coincided with the development of mechanical allodynia, and treatment with nefopam effectively decreased the NK1 receptor concentration and increased the PWT. These results have interesting applications for better understanding of the mechanism of

vincristine-related peripheral neuropathy and the relationship between nefopam and the NK1 receptor.

Data Sharing

The authors are willing to share the data in this article. The data can be accessible by contacting the corresponding author. These will be sent by means of the attached files.

Acknowledgment

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (grant number. 2017R1D1A1B03028360)

Author Contributions

JYL and HJP designed the study, analyzed data, and wrote the manuscript. NRC and BWK collected data. WSS and JYM contributed to the data analysis and revision. All authors contributed to data analysis, drafting or revision of the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no competing interests.

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