Treatment of electrical wrist stimulation reduces chemotherapy-induced neuropathy and ultrasound vocalization via modulation of spinal NR2B phosphorylation

Suk-Yun Kang, Se Kyun Bang, O Sang Kwon, Su-Yeon Seo, Kwang-Ho Choi, Seong Jin Cho, Hwa Seung Yoo, Jin Sun Lee, Hyun-Woo Kim, Yeonhee Ryu

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ABSTRACT

Docetaxel, a chemotherapeutic agent used to treat breast cancer, produces a robust painful neuropathy that is aggravated by mechanical and thermal stimuli. This study was undertaken to investigate the analgesic effects of electrical stimulation on docetaxel-induced neuropathic pain in mice and to identify associated changes in ultrasound vocalizations. Peripheral neuropathy was induced with intraperitoneally injected docetaxel (5 mg/kg) on 3 times every 2 days in male ICR mice. Electrical wrist stimulation was administered and pain behavior signs were evaluated by von Frey filaments and thermal stimulation on the hind paw. Ultrasound vocalizations were measured using ultrasound microphones, after electrical stimulation. After mice developed docetaxel-induced neuropathic pain behavior, an electrical stimulation temporarily attenuated mechanical allodynia and thermal hyperalgesia. In formalin and NMDA test, pain-induced mice showed increases in 10−30 kHz ultrasound vocalizations, but not in 30–50 and 50−80 kHz vocalizations. Treatment with docetaxel selectively increased 10−30 kHz ultrasound vocalizations, whereas electrical stimulation caused a meaningful decrease. Moreover, electrical stimulation suppressed the docetaxel-enhanced phosphorylation of the NMDA receptor NR2B subunit in the spinal dorsal horn. These results of the analgesic effect of electrical stimulation in chemotherapy-induced neuropathy could potentially provide a new method to treat and manage peripheral neuropathy in patients with cancer.

1. Introduction

The incidence of cancer has increased dramatically in recent years; thus, cancer-related pain has become increasingly common. Drugs that are used in chemotherapy to treat cancer can cause peripheral neuropathic pain, as well as psychological distress and sleep disorders. (Hong et al., 2014; Yeo et al., 2016) Chemotherapy-induced peripheral neuropathy (CIPN) is commonly associated with a sensory peripheral neuropathy with symmetrical symptoms that include numbness; loss of proprioception; tingling; and hyperalgesia or allodynia in the hands or feet. (Park et al., 2013) Although many medications are effective for pain management, there is no successful prevention or treatment of CIPN, and a number of patients suffer from pain even after the end of chemotherapy. (Stubblefield et al., 2009) CIPN is the most well-known side effect of chemotherapy and is caused by peripheral nerve damage following exposure to a neurotoxic chemotherapeutic agent (e.g., taxanes, platinum agents, vinca alkaloids, epothilones and bortezomib). (Hershman et al., 2014) Taxanes bind to β-tubulin of microtubules, interrupt axonal transport, target the sensory cell bodies and nerve axons, to induce neuronal cell death, (Bennett, 2010) while platinum compounds, which bind irreversibly to the DNA, induce apoptosis of sensory neurons. (von Schlippe et al., 2001) Docetaxel (DTX) is one of the most effective semi-synthetic taxane anticancer agents and is widely used for solid tumors such as breast, ovarian and non-small cell lung
carcinomas. (Cavaletti and Marmirol, 2010) However, DTX typically causes a sensory neuropathy, comprising paresthesia, numbness, or neuropathic pain in the hands or feet. Deficits in motor function are less common, but can present in severe cases. (Cervellini et al., 2010; Ventzel et al., 2016) DTX-induced neuropathy is less frequent and milder than paclitaxel-induced neuropathy. Considering the wide use of DTX and its cumulative neurological effects, neuropathy caused by the drug deserves significant attention. (Peng et al., 2012)

Ultrasound vocalizations (USVs) are an important form of communication, in addition to auditory squeaks, among animals and vocalizations above the frequency that humans can hear. (Portfors, 2007) Mouse USVs have been suggested as an effectual indicator for measuring the negative affective components of chronic cancer-related pain and neuropathic pain. (Kurejova et al., 2010) Adult rodents produce two different types of USVs, which appear to reflect the animal’s emotional state. USVs at the 50 kHz frequency tend to be produced in non-aggressive non-specific social interactions, such as playing or mating behavior in rodents. (Knutson et al., 1998; McGinnis and Vakulenko, 2003) In contrast, USVs at the 22 kHz frequency have been suggested to serve as alarm calls: warning colleagues of the presence of predators and/or informing them of the vocalizing animal’s pain and anxiety-related status. (Blanchard et al., 1991; Miczek et al., 1995)

On the other hand, it is well known that the activation of the N-methyl-D-aspartate (NMDA) receptor in the spinal cord plays a critical role in the induction of central sensitization, and phosphorylation of the NMDA receptor is closely related to the regulation of NMDA receptor function. (Zhou et al., 2011) Previous studies have shown that electrical stimulation in specific frequencies applied to certain acupuncture points can facilitate the release of specific neuropeptides in the central nervous system (CNS). (Han, 2003) Compared to high frequency, the use of low frequency electrical stimulation significantly decreased peripheral neuropathic pain in animal model. (Yu et al., 2017)

In this study, we aimed to determine whether electrical low frequency stimulation (LFS) at the wrist reduces DTX-induced peripheral neuropathic pain responses, such as mechanical allodynia and thermal hyperalgesia. We also measured USVs using a traditional acute control model, the formalin and NMDA test, to examine the effect of pain in specific ranges of USVs, and tested the effect of LFS on USVs indicative of pain. Furthermore, we evaluated whether these anti-nociceptive effects were related to the inhibition of spinal NMDA receptor excitability, as measured by the phosphorylation of NR2B subunit (pNR2B) expression in the spinal dorsal horn.

2. Materials and methods

2.1. Animals

All experiments were performed using male ICR mice (20–25 g; Samtako, Osan, Korea) housed in colony cages with free access to food and water, and maintained in a standard environment consisting of a 12 h light/dark cycle with lights on at 07:00, as well as constant room temperature and humidity (24 ± 2 °C, 40–60 %), for at least 1 week before the experiment. All experiments were conducted in accordance with the ethical guidelines of the International Association for the Study of Pain and were approved by the Animal Care and Use Committee at the Korea Institutes of Oriental Medicine (KIOM) with reference number #16–062. To examine the DTX-induced pain behaviors, animals (total n = 24) were randomly divided into 3 treatment groups as follows: normal (Normal, n = 8), vehicle (5% DMSO, n = 8) and DTX (DTX5 mg/kg, n = 8), to examine the analgesic effect of LFS, animals (total n = 24) were randomly divided into 3 treatment groups as follows: DTX (DTX, n = 8), anesthesia without LFS treatment [DTX + (Anes), n = 8] and anesthesia with LFS treatment [DTX + LFS(Anes), n = 8] and to examine the change of USVs, animals (total n = 20) were randomly divided into 5 treatment groups as follows: normal (Normal, n = 4), NMDA injection (NMDA, n = 4), formalin injection (formalin, n = 4), DTX (DTX, n = 4) and LFS treatment (DTX + LFS, n = 4).

2.2. DTX administration

Docetaxel (DTX, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 5% dimethylsulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 2 mg/mL depending on animal weight, and animals treated intraperitoneally at a dose of 5 mg/kg/day for 3 times every 2 days. The vehicle control animals received an equivalent volume of 5% DMSO via the same injection route.

2.3. Electrical low frequency stimulation (LFS) treatment

Prior to LFS treatment, all mice were transiently anesthetized with 3% isoflurane in a mixture of N2O/O2 gas to reduce handling-induced stress. Animals were placed on the thermostatic pad and then an electric stimulation plate (2 × 5 mm) was attached around the Neiguan (PC6) acupuncture point through the median nerve on the right wrist; an additional stimulation plate was attached to the middle of ipsilateral paw (palp). PC6 point is located between the tendons of them, palmaris longus and flexor carpi radialis, proximal to the transverse crease of the wrist between the tendons of them. LFS treatments were administered by trained experts using the Morning band (Piomed Inc., Seoul, Korea) with a stimulation intensity of 800 μA and frequency of 16 Hz (0.1 ms biphasic alternating current pulse width) for 10 min. The intensity was adjusted to maintain slight twitching of the local muscles, indicating satisfactory stimulation.

2.4. Evaluation of pain behavior

To assess DTX-induced peripheral neuropathic pain responses, such as mechanical allodynia and thermal hyperalgesia, we measured paw withdrawal response using a von Frey filament (0.4 g, North Coast Medical, Morgan Hill, CA, USA) and plantar analgesia meter (IITC Life Science Inc., Woodland Hills, CA, USA), as described previously. (Choi et al., 2015)

Briefly, normal baseline values of the withdrawal response to mechanical or heat stimulation were measured prior to DTX treatment. The paw withdrawal response frequency (PWF) to normally innocuous mechanical stimuli were measured using a von Frey filament with a force of 0.4 g. Mice were placed on a metal mesh grid under a plastic chamber, and the von Frey filament was applied from underneath the metal mesh flooring to each plantar of the hind paw.

The von Frey filament was applied 10 times to each hind paw, and the number of paw withdrawals responses out of 10 was then counted. The results of mechanical behavioral testing in each experimental animal were expressed as a percent withdrawal response frequency (PWF, %), which represents the percentage of paw withdrawals out of the maximum of 10.

To determine noxious responses to heat stimuli, animals were placed in a plastic chamber (15 cm in diameter; 20 cm in length) on a glass floor and allowed to acclimate for 10 min before thermal hyperalgesia testing. A radiant heat source was positioned under the glass floor beneath each hind paw, and paw withdrawal latency (WRL) was measured to the nearest 0.1 s using a plantar analgesia meter. The cut-off time was set at 20 s to prevent possible tissue damage. All behavioral tests were conducted by an experimenter who was blinded to the treatment condition.

2.5. Ultrasound vocalizations (USVs)

In order to measure spontaneous pain induced by DTX-induced peripheral neuropathic pain, the audible USVs was measured. Prior to USVs measurement, individual mice were placed in an experimental cage without bedding and adapted 30 min a day for 3 consecutive days to avoid stresses by environmental changes. High performance
ultrasound microphones (CM16/CMPA, 10–200 kHz frequency range, Avisoft Bioacoustics, Berlin, Germany) were positioned 30 cm above the experimental cage. To investigate the range of pain sounds uttered in each USV range, recording of USVs was performed following division of USVs into 10–30 kHz, 30–50 kHz, and 50–80 kHz ranges using an analysis program (Avisoft SASLab Pro, Avisoft Bioacoustics, Berlin, Germany). All experimental procedures were conducted between 15:00 and 18:00.

2.6. Formalin and NMDA-induced USVs

The formalin test was performed as previously described. (Kang et al., 2011) Mice were first acclimated for 30 min in an observation chamber, then formalin (1%, 20-μL) was injected subcutaneously into the plantar surface of the right hind paw with a 30-gauge needle. Following formalin injection, the animals were immediately returned to the observation chamber and USVs were recorded using an ultrasound microphone for 20 min.

Each mouse was acclimated in an observation chamber for at least 30 min before NMDA (0.4 nmol in 5-μL sterile saline) injection. Intrathecal (i.t.) injections of NMDA were performed according to the procedure reported in a previous study, (Choi et al., 2017) using a 10-μL Hamilton syringe with a 30-gauge needle. Briefly, the mouse was held tightly between the thumb and middle finger at the level of the both iliac crests, and the fifth lumbar spinal process was palpated with the index finger. The needle was inserted through the vertebral column into the lumbar 5 to lumbar 6 intervertebral space and a tail flick response was considered indicative of a successful i.t. injections. The NMDA was slowly injected over a 10-second period. Then, the needle was carefully removed from the spinal cord. Following injection, animals were immediately returned to the observation chamber and USVs were recorded using an ultrasound microphone for 10 min.

2.7. Immunohistochemistry and image analysis for pNR2B

Mouse (total n = 24) were euthanized at the endpoint of each treatment (21 days after DTX injection), and the lumbar 4 to lumbar 6 segments of the spinal cord were removed for pNR2B immunohistochemistry. Two hours after administration of electrical LFS, animals were deeply anesthetized with 5% isoflurane and perfused transcardially with calcium-free Tyrode solution followed by a fixative containing 4% paraformaldehyde. The spinal cord postfixed in the same fixative for 4 h, and then cryoprotected in 30% sucrose in phosphate-buffered saline for 48 h. Forty-micrometer thick transverse frozen sections were cut through the spinal cord using a cryostat. After elimination of endogenous peroxidase activity with 0.3% hydrogen peroxide and preblocking with 3% normal goat serum in phosphate-buffered saline for 48 h, Forty-micrometer thick transverse frozen sections were cut through the spinal cord using a cryostat. After elimination of endogenous peroxidase activity with 0.3% hydrogen peroxide and preblocking with 3% normal goat serum in phosphate-buffered saline, the free-floating sections through the lumbar spinal cord were incubated in rabbit anti-pNR2B antibody (1: 500, cat# PA5-37591, Invitrogen, CA, USA) at 4°C for 48 h. Subsequently, the sections were washed and then incubated in biotinylated goat anti-rabbit IgG (1:200; Vector, Burlingame, CA, USA), and the sections were then processed using avidin–biotin–peroxidase as previously described. (Clark et al., 2009) Finally, visualization was performed using 3,3-diaminobenzidine (DAB; Sigma) with 0.2% nickel chloride intensification.

For quantitative analysis of pNR2B-immunoreactive (ir) neurons in lumbar spinal dorsal horn, five spinal cord sections from the lumbar 4–lumbar 6 spinal cord segments were randomly selected from each animal and subsequently scanned. Individual sections were digitized with 4096 Gv levels using a cooled CCD camera (Micromax Kodak 1317; Princeton Instruments, AZ, USA) connected to a computer-assisted image analysis system (MetaMorph; Universal Imaging, PA, USA). To maintain a constant threshold for each image and to compensate for subtle variability of the immunostaining, we counted neurons that were at least 70% darker than the average gray level of each image after background subtraction and shading correction. The average number of pNR2B-ir neurons per section from each animal was obtained and these values were averaged across each group and presented as group data. The expression of pNR2B was quantified in the following three spinal cord dorsal horn regions: (1) the superficial dorsal horn (SDH, laminae I and II), (2) the nucleus proprius (NP, laminae III and IV), and (3) the neck region (NECK, laminae V and VI). These regions were identified based on cytoarchitectonic criteria as defined by previous report. (Abbadie and Besson, 1994) All pNR2B analysis procedures described above were performed blindly with regard to the experimental condition of each animal.

2.8. Data analysis

The sample size was calculated on the basis of the primary outcome of our study, which was differences in the mechanical paw withdrawal frequency among the experimental groups. All values are expressed as the mean ± SD. Statistical analysis was performed using Prism 5.0 (GraphPad Software, CA, USA). Repeated-measures two-way analysis of variance (ANOVA) was performed to determine overall effects; post hoc analysis was performed using Tukey’s multiple comparisons test to determine the p-value among the experimental groups. A value of p < 0.05 was considered to be statistically significant Fig. 1.

3. Results

3.1. Development of pain behavior by DTX

Multiple treatments of DTX (5 mg/kg, once daily) for 3 times every 2 days produced significant mechanical allodynia and thermal hyperalgesia, as shown in Figs. 2 and 3 (*p < 0.05, **p < 0.01, and ***p < 0.001 vs. normal). In both hind paws, whereas vehicle-treated animals (5% DMSO) did not change paw withdrawal frequency (%) compared with normal animals (Normal) from the first day of DTX administration to the last behavioral measurement on day 28, the DTX administration group exhibited increased paw withdrawal frequency (%), compared with the frequency in normal animals, from day 7 to day 28 after DTX administration [Figs. 2 (A) and (B)]. Furthermore, the vehicle group did not exhibit changes in paw withdrawal latency (sec) to the thermal stimuli for all days of behavioral measurements, compared with the latency in normal animals. The paw withdrawal latency of DTX-treated animals in the right hind paw decreased significantly from day 14 to day 28, and the paw withdrawal latency in the left hind paw decreased from day 7 to day 28, compared with the latency in normal animals [Figs. 3 (A) and (B)].

3.2. Anti-nociceptive effect of single LFS administration

LFS administration temporarily produced anti-nociceptive effects on DTX-induced mechanical allodynia and thermal hyperalgesia, compared with DTX-treated neuropathic mice (Figs. 4, *p < 0.05 and **p < 0.01). Because the pain behavior was well maintained at 3 weeks
after DTX treatment, we applied LFS at day 21. LFS-treated animals (DTX + LFS) exhibited significant reductions in paw withdrawal frequency (%) at 1 h after LFS administration, compared with DTX-induced neuropathy animals [DTX, Fig. 4 (A)]. Regarding paw withdrawal latency (sec) to the thermal stimuli, LFS-treated animals (DTX + LFS) exhibited anti-hyperalgesic effects at 1 and 2 h after LFS administration, compared with DTX-treated animals [DTX, Fig. 4 (B)].

3.3. Changes in ultrasonic vocalizations (USVs) in NMDA and formalin animal models

To determine the frequencies of USVs that mice utter when they feel pain, we measured USVs using an NMDA and formalin test. Animals that were stimulated by spinal and peripheral pain induction methods (NMDA and formalin) produced many USVs in the 10–30 kHz range, compared with the USVs of normal mice (Fig. 5, *p < 0.05 and ***p < 0.001). Animals intrathecally treated with NMDA exhibited significant increases in USVs within the 10–30 kHz range, but not the 30–50 and 50–80 kHz ranges, compared with normal animals. For peripheral nerve stimulation, animals treated with formalin in the paw exhibited significant increases in USVs within the 10–30 kHz range, similar to the results of the NMDA test.

3.4. Changes in ultrasonic vocalizations (USVs) in single LFS administration

Because mice showed the greatest increase in USVs in the 10–30 kHz range when they felt pain, we investigated how USVs in the 10–30 kHz range changed in DTX-induced neuropathic pain mice. We performed this experiment on day 21 after DTX treatment, a time point when pain was well maintained. While animals with DTX-induced peripheral neuropathic pain exhibited increased USVs, animals that underwent LFS administration exhibited reduced USVs, compared with USVs in normal mice (Fig. 6, *p < 0.01 and ***p < 0.001). The DTX-induced peripheral neuropathy mice (DTX) exhibited significant increases in USVs within the 10–30 kHz range, compared with the normal group (Normal), whereas neuropathy mice that underwent a single LFS treatment (DTX + LFS) exhibited decreases in USVs, compared with neuropathy mice.

3.5. Effect of LFS administration on pNR2B expression in the spinal dorsal horn

As compared with normal animals, vehicle-injected animals (5% DMSO) did not change the number of pNR2B-ir neurons in any of the 3 spinal cord dorsal horn regions (SDH, NP, and NECK). Animals of DTX-treated (DTX) remarkably increased the number of pNR2B-positive neurons in all 3 dorsal horn regions as compared with normal (Normal, **P < .01, and ***P < .001) and vehicle group (++P < .01, and +++P < .001 in Fig. 7A). Although DTX animals that is only anesthetized and not administered LFS (DTX + (Anes)) did not change the number of DTX-induced pNR2B-ir neurons in any of the 3 spinal cord dorsal horn regions examined as compared with DTX-treated group (DTX), group of DTX animals that administrated LFS [(DTX + LFS (Anes)] demonstrated a significant decrease in the number of pNR2B-ir neurons in NP and NECK regions as compared with DTX-treated animals. (++P < .05 and +++P < .01 in Fig. 7A). Fig. 7B illustrates representative photomicrographs of spinal pNR2B-ir neurons from normal animals (b), from DTX-treated animals (c), and from DTX animals that received electrical LFS-treatment (d). Panel (a) in Fig. 7B shows a diagram depicting the location of the different regions of the spinal cord dorsal horn that were analyzed in this study, which includes the superficial dorsal horn (SDH, laminae II–I), the nucleus proprius (NP, laminae III–IV) and the neck of dorsal horn (NECK, laminae V–I).

4. Discussion

A previous study from our laboratories showed that low frequency electro-acupuncture stimulation at the ST36 acupuncture point (also
known as “Joksanmni”) suppressed paclitaxel-induced peripheral neuropathic pain and spinal expression of pNR2B via the mediation of spinal opioid receptor, alpha2-adrenoceptor, and beta-adrenoceptor activities in mice. (Choi et al., 2015) The present study demonstrated that repetitive injections of DTX produced peripheral neuropathic pain responses (mechanical allodynia and thermal hyperalgesia) and that LFS administration on the wrist suppressed DTX-induced peripheral neuropathic pain in mice. In a further experiment, we demonstrated that, using traditional acute pain models, USVs of animals were increased in the 10–30 kHz range when they perceived pain; moreover, DTX-induced USVs in the 10–30 kHz range were reduced by LFS administration.

CIPN, the most well-known side effect of chemotherapy, is defined as peripheral nerve damage caused by exposure to a chemotherapeutic drug with neurotoxicity. It is a common non-hematologic adverse effect of chemotherapy and appears to be a purely sensory peripheral neuropathy with symmetrical symptoms, typically including numbness, loss of proprioceptive sense, tingling, pins and needles sensations, hyperalgesia, or allodynia in the hands or feet. (Park et al., 2013) CIPN is a common cause for dose reduction or even termination of chemotherapy; however, 30 % of patients with CIPN continue to suffer from symptoms at ≥6 months after the end of their chemotherapy. (Seretny et al., 2014) In general, CIPN is associated with treatment by multiple chemotherapeutic agents, including platinum compounds (cisplatin, carboplatin, oxaliplatin), taxanes (paclitaxel, docetaxel), vinca alkaloids (vincristine, vinblastine), thalidomide, and bortezomib. (Park et al., 2013)

DTX-induced peripheral neuropathy is less frequent and milder than the neuropathy induced by paclitaxel. (Ko et al., 2017) In light of the many uses and excellent effects of DTX, prevention and management of associated peripheral neuropathy requires considerable effort. (Brewer et al., 2016) This study showed that repetitive administration of DTX

Fig. 4. Graphs show the anti-nociceptive effect of single low frequency stimulation (LFS) administration on mechanical allodynia (A) and thermal hyperalgesia (B) in docetaxel (DTX)-induced neuropathic mice. The LFS administration group (DTX + LFS, n = 8) exhibited significant suppression of DTX-induced mechanical allodynia within two hours. In studies of thermal hyperalgesia, paw withdrawal latency increased from 2 h to 3 h after LFS treatment, compared with latency in DTX-induced neuropathic mice (DTX, n = 8, *P < 0.05 and **P < 0.01).

Fig. 5. Graph and representative photos showing reductions in ultrasonic vocalizations (USVs) in two typical animal models of pain. In the animal models of pain (NMDA and formalin tests, n = 4 each), USVs in the 10–30 kHz range were produced significantly more often than in normal animals (n = 4, *P < 0.05 and ***P < 0.001). USVs in the 30–50 and 50–80 kHz ranges showed no difference. Photographs (a), (b) and (c) are representative USVs pictures of normal, NMDA injection and formalin injection animal.
produces significant peripheral neuropathic pain behaviors (mechanical allodynia and thermal hyperalgesia) 7 days after the first treatment of DTX, which are sustained for 4 weeks. Taxane-based chemotherapeutic agents cause peripheral neuropathic pain, the risk of which increases with the administration of additional doses. (Cervellini et al., 2010; Ventzel et al., 2016) Neuropathic pain can be induced by various etiologic agents, such as nerve trauma, metabolic disease, infection, or tumor; however, the precise pathophysiology of neuropathic pain
remains unclear. (Jay and Barkin, 2014). Unfortunately, there are no known drugs that successfully prevent CIPN, and discovery of drugs that aid in the treatment of CIPN has been limited. (Hershman et al., 2014) Contemporary treatment modalities are mainly designed to control neuropathic pain symptoms, using pharmaceutical agents that often are restricted in use due to side effects.

Invasive interventions, such as sympathetic nerve blocks and implantable spinal or peripheral nerve stimulators, are rarely considered. Transcutaneous electrical nerve stimulation (TENS), one of the non-invasive methods, has been shown to directly impact the onset of pain using an electric field, although the precise mechanism of action has not yet been elucidated, TENS is known to reduce the pain signal through the central nervous system. (Bennett et al., 2016; Cho et al., 2014; Choi et al., 2015) Transcutaneous electrical acupuncture point stimulation (TEAS) is also a non-invasive electrical stimulation that produces a perceptible sensation via electrodes attached to the skin, and it has no risk of needle induced contagious disease or infection compared to acupuncture. (Wang et al., 2014) The frequency of electrical pulses is one of the crucial factors of duration and analgesic effect provided by electrical stimulation. (Johnson and Martinson, 2007) Electrical stimulation at 2, 15 and 100 Hz frequencies significantly reduces pain sensation, but each of these stimulation frequencies appears to suppress pain sensation by the selective activation of different neuronal pathways. (Han, 2004) The anti-inflammatory effects of 2 Hz electrical acupuncture on inflammatory pain animals were mediated by sympathetic post-ganglionic neurons, while the effects of 100 Hz electrical acupuncture were mediated by the sympathetic-adrenal medullary axis, (Kim et al., 2008) and opioid-related substances injected into the cerebral ventricle dose-dependently reduced the analgesia induced by 2 Hz electrical acupuncture, but not that induced by 100 Hz electrical acupuncture. (Jiang et al., 2006) In other study, TEAS at low frequency enhanced μ opioid receptors expression in the L3-L5 dorsal root ganglion, which mediate the analgesic effect, but not high frequency TEAS. (Yu et al., 2017) Many studies have shown that electrical stimulation at a specific frequency can promote the release of specific neuropeptides from the CNS and that low-frequency stimulation is more analgesic than high-frequency. This study showed that a single 16 Hz LFS treatment at the PC6 acupuncture point produced a temporary analgesic effect in the DTX-induced peripheral neuropathic pain behaviors, suggesting the possibility of more persistent and powerful analgesic effects following repetitive LFS treatment. Similar to our results, oxaliplatin-induced peripheral neuropathy in patients with gastrointestinal cancer exhibited significant improvements in neurotoxic symptoms, following laser acupuncture treatment at the PC6 acupuncture point. (Hsieh et al., 2016) In addition, Qiao et al. reported that the application of electro-acupuncture to the PC6 point in rats with incisional neck pain increased the thermal pain threshold. (Qiao et al., 2017)

As USVs are an important form of communication in rodents and a relatively easily quantified measure, the analgesic effects of LFS on DTX-induced USVs were confirmed using ultrasonic microphones. Litvin et al. showed that rats produce two distinct types of USVs, which appear to reflect the animal's emotional state: either a positive state (50 kHz USVs) or a negative state (22 kHz USVs). (Litvin et al., 2007) While the 22 kHz USVs have been commonly suggested as a measure of affective shifts in rats, (Knutson et al., 2002) the 50 kHz USVs tend to be produced in non-aggressive conspecific social interactions and during play. (McGinnis and Vakulenko, 2003) USVs have been postulated as an indicator of on-going pain and have thus been suggested as a potential method for measuring the negative affective components of pain. USVs produced by experimental rats in response to pain conditions provide more information regarding their acute emotional state than do their audible vocalizations; (Takahashi et al., 2010) however, in mice this may not always be the case. While 50 kHz USVs are elicited robustly during mating behavior in rats, (McGinnis and Vakulenko, 2003) other studies have shown that 50 kHz USVs are emitted in response to pain stimulation in mice. (Kurejova et al., 2010; Tsuzuki et al., 2012) Therefore, we employed the traditional acute pain models (i.e., the formalin and NMDA tests) to determine which ranges of USVs are produced when mice feel pain. We found that, upon application of nociceptive irritants, such as formalin and NMDA, mice produced more USVs in the 10–30 kHz range, compared with the 30–50 and 50–80 kHz ranges. Thus, we demonstrated that the USV range of the animals when they feel pain is within 10–30 kHz. To determine how USVs in the 10–30 kHz range are changed by LFS, we measured USVs immediately after the administration of LFS in DTX-induced peripheral neuropathy mice. Animals treated with DTX for 3 times every 2 days exhibited an increase in USVs in the 10–30 kHz range, while animals that underwent LFS administration revealed a meaningful decrease in the same USVs. These results were similar to our pain behavior test results; moreover, they supported previous reports, which showed that adult rats emit low range USVs that have a relatively low peak frequency (20–30 kHz) in dangerous situations, such as predator exposure or fighting (these are often termed “22 kHz calls”. (Inagaki and Ushida, 2017; Lim et al., 2018) These 22 kHz calls have been suggested to serve as alarm calls, informing other animals of the vocalizing caller's anxiety and negative state. (Miczek et al., 1995)

In addition, the present study examined whether the analgesic effect of electrical LFS administration is associated with the suppression of spinal NMDA receptor activity, as measured by a reduction in pNR2B neurons in the spinal cord dorsal horn in DTX-induced peripheral neuropathy mice. It has been reported that spinal NMDA receptors play an important role in central sensitization and are closely linked to the development and maintenance of pain behavior under cancer-chemotherapeutic drugs-induced peripheral neuropathic conditions. (Jaggi and Singh, 2012; Jay and Barkin, 2014) In particular, an increase in NR2B has been recognized as a major mechanism underlying the regulation of NMDA receptor function. An activation of spinal NR2B protein and mRNA in oxaliplatin-induced neuropathy is reported during late phase, but not in early phase, and selective NR2B antagonists significantly attenuated the oxaliplatin-induced pain behavior, suggesting the critical role of spinal NR2B-containing NMDA receptors in chemotherapy-induced neuropathy. (Mihara et al., 2011) A recent study from our laboratories has also demonstrated that electro-acupuncture stimulation into the acupuncture point produced an inhibitory effect on paclitaxel-induced peripheral neuropathy and decreased the expression of phosphorylation of spinal NR2B level. (Choi et al., 2015)

In conclusion, the present study demonstrated that administration of LFS on the wrist significantly attenuated DTX-induced mechanical allodynia and thermal hyperalgesia in neuropathic mice. LFS also suppressed DTX-increased USVs in the 10–30 kHz range, a typical USV range used when mice feel pain. Collectively, the results of this study suggest that LFS can be a potential strategy for the management of chemotherapeutic drug-induced neuropathic pain in cancer patients. Although this study has been performed in a well-designed manner in an animal model, further clinical studies are needed to confirm the analgesic effects of low frequency stimulation.

Author contributions

S-Y.K. designed and performed experiments and prepared the manuscript. S.K.B. and O.S.K. gave technical support, guided the design of the study and drafting of the manuscript. S-Y.S., K-H.C. and, S.J.C. gave technical support in experiments, helped with analysis, interpretation of data and drafting of the manuscript. H.S.Y., J.S.L., H-W.K. and Y.R. gave conceptual advice and assisted with the study design, interpretation of the data and the writing of the manuscript. All authors have read and approved the final manuscript.
Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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