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An effective therapeutic approach for oxaliplatin-induced peripheral neuropathy using a combination therapy with goshajinkigan and bushi

Keita Mizuno[a], Keisuke Shibata[b], Ryohei Komatsu[b], Yuji Omiya[a], Yoshio Kase[a], and Schuichi Koizumi[b]

[a]Tsumura Research Laboratories, Kampo Scientific Strategies Division, Tsumura & Co., Yoshiwara, Ami-machi, Inashiki-gun, Ibaraki, Japan; [b]Department of Neuropharmacology, Interdisciplinary Graduate School of Medicine, University of Yamanashi, Shimokato, Chuo, Yamanashi, Japan

ABSTRACT

Oxaliplatin-induced peripheral neuropathy (OIPN) occurs at extraordinarily high frequency, but no effective treatment for this disorder has been established. Goshajinkigan (GJG), a traditional Japanese medicine known as *Kampo*, is known to reduce OIPN in both basic and clinical studies. However, its molecular mechanisms remain largely unknown. Here, we elucidate the mechanisms underlying the therapeutic effects of GJG against OIPN and the therapeutic benefits of combining GJG with bushi, a herbal medicine derived from the processed *Aconiti tuber*. Oxaliplatin (4 mg/kg) was injected into mice twice a week for up to 4 and 3 weeks, respectively. OIPN was assessed using pain behavioral tests, such as those testing cold hypersensitivity, thermal hyperalgesia, and mechanical allodynia, as well as a reduction of the current perception threshold (CPT). GJG (0.3 or 1 g/kg) and bushi (0.1 or 0.3 g/kg) were orally administered 5 times a week for 4 weeks. Behavioral analysis was performed 24 h after the final dose.

Oxaliplatin induced cold hypersensitivity and mechanical allodynia but not thermal hyperalgesia and reduced CPT of Aδ- and Aβ-fibers but not C-fibers. All these effects were counteracted by GJG. Bushi, an ingredient of GJG that shows analgesic effect, reduced oxaliplatin-induced cold hypersensitivity but had no effect on oxaliplatin-induced mechanical allodynia. However, bushi significantly accentuated the effects of GJG when co-administered with GJG. GJG reduces OIPN by counteracting the sensitization of Aδ- and Aβ-fibers and shows analgesic effects against cold hypersensitivity and mechanical allodynia. These effects are potentiated by bushi. The combination of GJG with bushi has high potential for preventing OIPN.

Abbreviations: CPT, Current perception threshold; GJG, Goshajinkigan; OIPN, Oxaliplatin-induced peripheral neuropathy; TRP channels, Transient receptor potential channels

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Introduction

Oxaliplatin, a third-generation platinum drug, is widely used to treat advanced/recurrent colorectal cancer.[1-3] Oxaliplatin causes neuropathy as an adverse effect with an extraordinarily high frequency, which is viewed with suspicion because it is the most common dose-limiting factor for oxaliplatin therapy.[4-6] The acute phase of oxaliplatin-induced peripheral neuropathy (OIPN) is characterized by cold hypersensitivity, which is followed by the chronic phase of OIPN characterized by sensory ataxia, numbness, and sensations of pain, such as mechanical allodynia. These symptoms of OIPN worsen with continued oxaliplatin therapy; therefore, the discontinuation of oxaliplatin therapy is frequently required, leading to a decrease in therapeutic effects. However, a limited number of OIPN treatments, such as calcium gluconate and magnesium sulfate,[7] analgesic drugs (pregabalin and opioids)[8,9] and antioxidants (silibinin and polyphenols, such as quercetin),[10,11] have been investigated in basic and clinical research, and they have not achieved satisfactory outcomes in clinical practice.

Goshajinkigan (GJG), a traditional Japanese herbal medicine known as *Kampo*, has been widely used in Japan to treat rhigosis or numbness in the extremities and disease-associated neuropathies, such as diabetic neuropathy.[12,13] Recent accumulating evidences in basic and clinical studies indicate that GJG improves OIPN.[14-17] Mizuno et al recently demonstrated in an acute OIPN rat model that GJG prevents oxaliplatin-induced cold hypersensitivity by suppressing cold-sensitive TRP channels, such as TRPA1 and TRPM8, in peripheral sensory neurons.[18] However, the mechanisms underlying the GJG-induced amelioration of OIPN, especially the cumulative neuropathy, remain unknown.

Bushi (TJ-3023, Tsumura & Co., Japan), a herbal medicine derived from the processed *Aconiti tuber*, is one of the ingredients of GJG and is well known to show an analgesic effect; [19,20] thus, it is used not only for chronic and persistent pain, including neuropathic pain, but also to potentiate the analgesic effects of some kinds of *Kampo* medicines in Japan.[21] Thus, bushi might potentiate the analgesic effects of GJG in OIPN when co-administered.

In the present study, we demonstrate that OIPN, manifested as cold hypersensitivity and mechanical allodynia, is associated with the sensitization of Aδ- and Aβ-fibers, which is inhibited by GJG in a mouse model. In addition, we demonstrate that GJG, when combined with bushi, dramatically increases analgesic effects,

CONTACT Schuichi Koizumi skoizumi@yamanashi.ac.jp Department of Neuropharmacology, Interdisciplinary Graduate School of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409 3898, Japan.

demonstrating that a combination therapy using GJG and bushi is a potential strategy for OIPN treatment.

Results

GJG inhibits oxaliplatin-induced cold hypersensitivity and mechanical allodynia but not thermal hyperalgesia in mice

The repeated injection of oxaliplatin (4 mg/kg) induced a sustained cold allodynia, measured as an increase in the duration of withdrawal responses to cold stimulation using the acetone test, in mice from day 3 after the first injection (Fig. 1A). Oxaliplatin also led to a persistent cold hyperalgesia, measured as a decrease in the latency of withdrawal responses using the cold plate test, from day 3 after the first injection (Fig. 1B). GJG (1 g/kg) ameliorated the oxaliplatin-induced cold hypersensitivity at all the time points tested (Fig. 1A and B). GJG ameliorated the cold allodynia in a concentration-dependent manner over a concentration range from 0.3 to 1 g/kg (Fig. 1E). However, oxaliplatin never induced thermal hyperalgesia, measured as a decrease in the latency of withdrawal

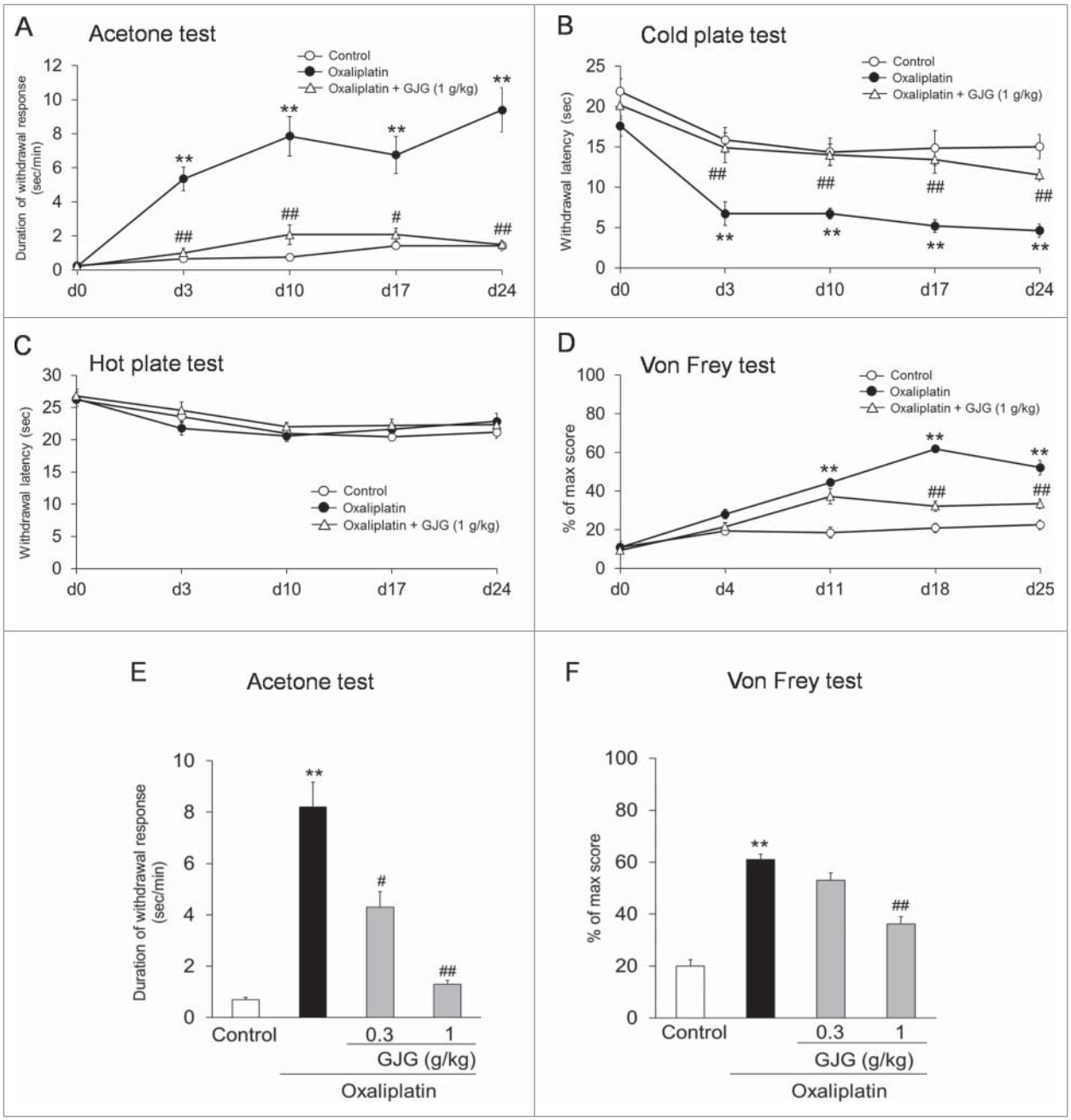

Figure 1. Goshajinkigan inhibits oxaliplatin-induced cold hypersensitivity and mechanical allodynia but not thermal hyperalgesia in mice. Oxaliplatin (4 mg/kg) was injected intraperitoneally twice a week for 4 weeks. Goshajinkigan (GJG) (0.3 or 1 g/kg) was administered orally, immediately after the injection of oxaliplatin, 5 times a week for 4 weeks. The acetone test was performed at the indicated periods (A) and on day 3 (E) to assess the effects of GJG on oxaliplatin-induced cold allodynia. The cold plate (B) and hot plate (C) tests were performed at the indicated periods. The latency of the withdrawal response against each thermal stimulus was measured. The von Frey test was performed to assess mechanical allodynia at the indicated periods (D) and on day 18 (F). The paw withdrawal response to the tactile stimulus was evaluated. d; days after oxaliplatin treatment; $^{**}P < 0.01$ compared with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the oxaliplatin-treated group. Data are expressed as the mean ± standard error of the mean. n = 5–6 per group.

responses using the hot plate test (Fig. 1C). As shown in Fig. 1D, oxaliplatin also caused mechanical allodynia, which had a slow onset and was significantly increased at day 11 and later after the first administration of oxaliplatin. GJG (1 g/kg) significantly inhibited the oxaliplatin-induced mechanical allodynia, but the lower dose of GJG (0.3 g/kg) had no effect (Fig. 1F).

The oxaliplatin-induced hyperactivation of Aδ- and Aβ-fibers, and its inhibition by GJG

The repeated injection of oxaliplatin had no effect on the threshold of the 5-Hz stimulus, which is a measure of the sensitivity of C-fibers (Fig. 2A and B). However, oxaliplatin significantly reduced the threshold of withdrawal responses to the 250- and 2000-Hz stimuli at day 12 and day 5, respectively, which lasted at least until day 26 (Fig. 2C and E). Thus, oxaliplatin increased the sensitivities of Aδ- and Aβ-fibers but not C-fibers. GJG (1 g/kg) significantly ameliorated these changes in sensitivity at all time points (Fig. 2C and E). The effects of GJG were not observed at the lower dose (0.3 g/kg) (Fig. 2D and F).

Bushi potentiated the ameliorating effects of GJG against OIPN

Bushi itself has analgesic effects; in fact, bushi (0.3 g/kg) alone reduced oxaliplatin-induced cold allodynia (Fig. 3A). When co-administered with GJG (0.3 g/kg), bushi significantly enhanced the anti-cold allodynic effects of GJG (Fig. 3C). In contrast, neither bushi (0.3 g/kg) alone nor GJG (0.3 g/kg) alone showed an analgesic effect against mechanical allodynia. However, when they were co-administered, they significantly inhibited oxaliplatin-induced mechanical allodynia (Fig. 3B and D).

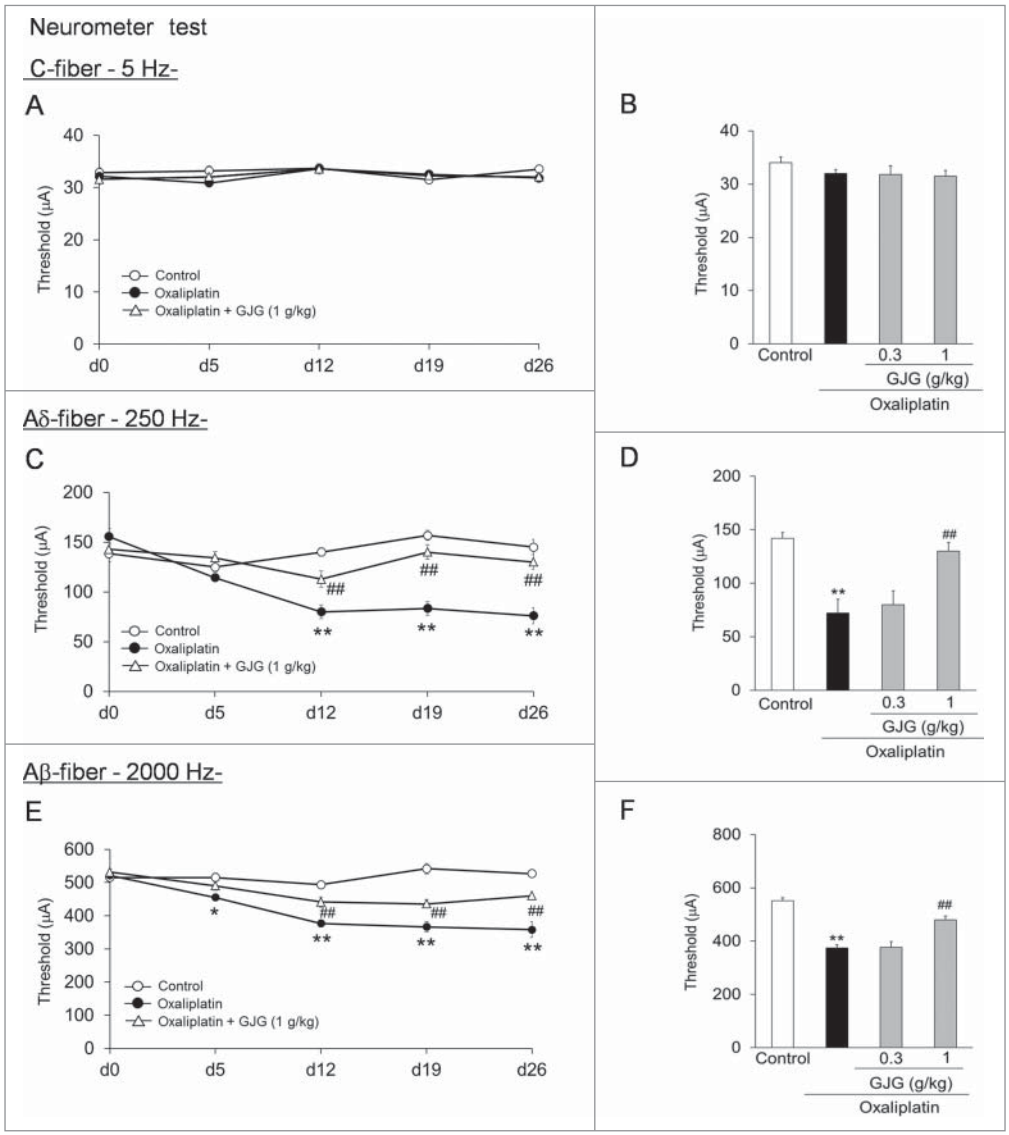

Figure 2. The oxaliplatin-induced hyperactivation of Aδ- and Aβ-fibers, and its inhibition by goshajinkigan. The sensitivities of different types of peripheral neurons (C-, Aδ-, and Aβ-fibers) were assessed using a neurometer test. The thresholds of paw withdrawal responses to electrical stimuli with 5 Hz (C-fibers, (A)and B), 250 Hz (Aδ-fibers, (C)and D), and 2000 Hz (Aβ-fibers, (E)and F) were measured. (A), (C), and (E) shows the time courses of sensitization of C-, Aδ-, and Aβ-fibers, respectively. (B), (D), and (F) show the concentration dependence of the effects of goshajinkigan on the sensitivity of C-, Aδ-, and Aβ-fibers, respectively on day 19. d, days after oxaliplatin treatment; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group; $^{\#\#}P < 0.01$ compared with the oxaliplatin-treated group. Data are expressed as the mean ± standard error of the mean. n = 5–6 per group.

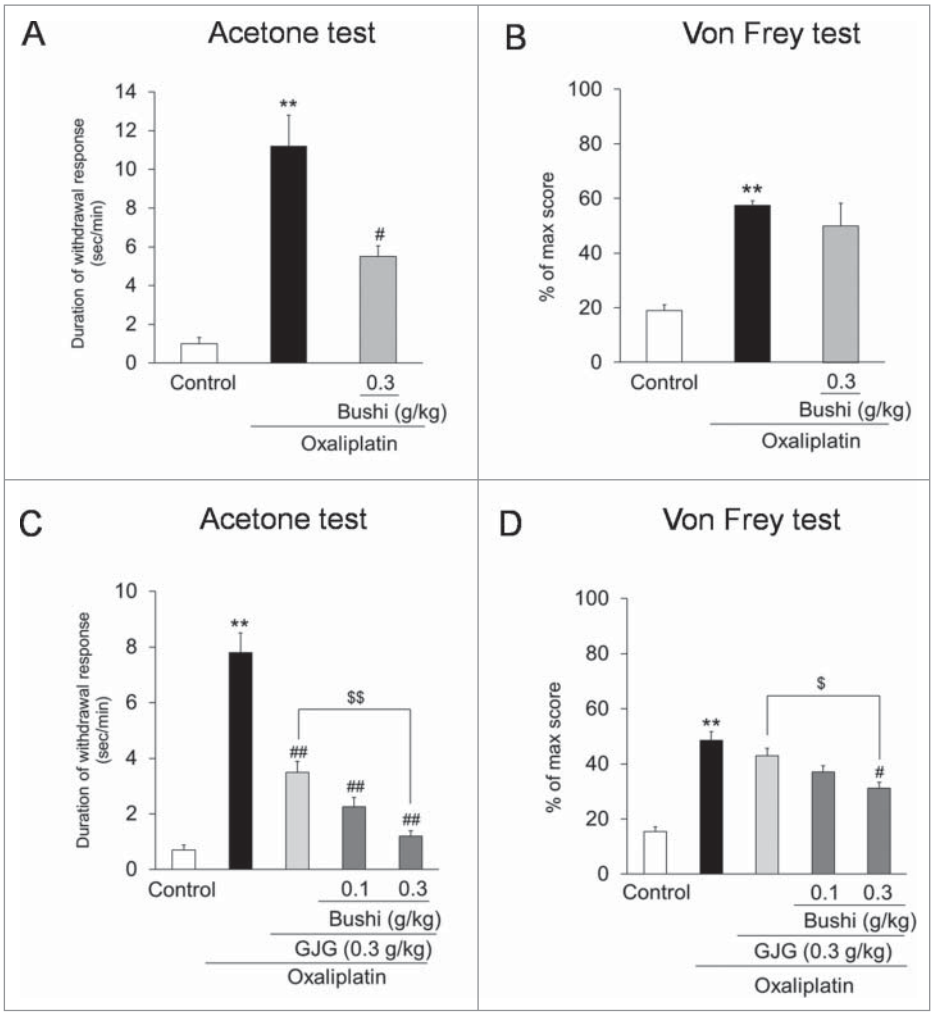

Figure 3. Bushi potentiates the ameliorating effects of goshajinkigan against oxaliplatin-induced peripheral neuropathy. Goshajinkigan (GJG) (0.3 g/kg) and/or bushi (0.1 or 0.3 g/kg) were administered orally, immediately after the injection of oxaliplatin, 5 times a week for 3 weeks. The acetone test was performed on day 3 ((A)and C). The von Frey test was performed on day 18 ((B) and D). $^{**}P < 0.01$ compared with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the oxaliplatin-treated group. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ compared with the oxaliplatin + GJG group. Data are expressed as the mean ± standard error of the mean. n = 10 per group.

Discussion

In this study, we demonstrated that OIPN is associated with the sensitization of Aδ- and Aβ-fibers, all of which were inhibited by GJG. Furthermore, we found that bushi, one of the ingredients of GJG that is thought to be important for the analgesic effects of GJG, significantly enhanced the therapeutic effects of GJG when used in combination with GJG. This suggests that a combination therapy using GJG and bushi is a potential strategy for OIPN treatment.

We initially characterized OIPN in mice and investigated the effects of GJG on OIPN. We found that oxaliplatin caused cold hypersensitivity in the early period (Fig. 1A and B), which was followed by mechanical allodynia (Fig. 1D). Interestingly, it did not cause thermal hyperalgesia (Fig. 1C). These characteristics of neuropathy in mice were similar to those seen in the clinical symptoms in human.[4,6] We also investigated the effects of oxaliplatin on the sensitivity of individual peripheral sensory neurons using a neurometer test[22] and found that oxaliplatin causes a significant increase in the responsiveness of myelinated peripheral sensory neurons, including Aδ- and Aβ-fibers, which sense fast pain and tactile pressure, respectively. However, oxaliplatin did not affect C-fibers (Fig. 2). The time course of sensitivity of these A-fibers was roughly the same as that of mechanical allodynia. Unlike nociceptive pain, mechanical allodynia starts with a sense of innocuous tactile stimuli by non-nociceptive neurons, such as Aβ-fibers, which somehow transmit tactile stimuli as pain in pathological conditions. The sensitization of Aβ-fibers, therefore, would be the most likely event responsible for oxaliplatin-induced mechanical allodynia. GJG significantly inhibited mechanical allodynia (Fig. 1D and F) and the sensitization of Aδ- and Aβ-fibers (Fig. 2C-F). In addition, Kono et al reported a morphological analysis demonstrated that oxaliplatin causes the atrophy of axons containing myelinated nerve fibers but not non-myelinated nerve fibers in the sciatic nerves in rats and that this effect was ameliorated by GJG.[17] The simplest interpretation of these findings is that an increase in the sensitivity of either Aδ- or Aβ-fibers or both is involved in the induction of mechanical allodynia and that GJG, acting on these sensory fibers, inhibits their sensitization.

As for heat sensation, the main heat sensor TRPV1 is almost extensively located in C-fibers.[23] This leads to the interpretation that oxaliplatin affects neither thermal hyperalgesia (Fig. 1C) nor the sensitivity of C-fibers (Fig. 2A and B). As for cold

sensation, the cold sensors TRPA1 and TRPM8 are functionally upregulated in dorsal root ganglion neurons after treatment with oxaliplatin.[18,24,25] TRPA1 is mainly colocalized with TRPV1-positive neurons, which are present in C-fibers, whereas TRPM8 is located in both C- and A-fibers.[23] Oxaliplatin, however, induced cold hypersensitivity despite it having no effect on the sensitivity of C-fibers in our study. Thus, the simplest interpretation of our present results is that in our model or administration schedule, oxaliplatin mainly acted on and sensitized TRPM8 in myelinated A-fibers, thereby leading to cold hypersensitivity. Alternatively, oxaliplatin may upregulate TRPA1 or molecules other than TRPA1 in A-fibers or may affect TRPM8 in A-fibers. Although the mechanisms underlying oxaliplatin-induced cold hypersensitivity are still unknown, we demonstrated that GJG significantly inhibited cold hypersensitivity (Fig. 1A, B and E) and the sensitization of Aδ- and Aβ-fibers (Fig. 2C-F). Considering the present results that oxaliplatin sensitized only myelinated A-fibers, where TRPA1 is not present,[23] GJG may mainly act on and inhibit TRPM8 in A-fibers and, thereby, inhibit cold hypersensitivities. However, further studies are needed to clarify this.

Another important finding is that bushi, one of the ingredients of GJG, significantly enhances the therapeutic effects of GJG, thus proposing a combination therapy using GJG and bushi. Bushi is widely used in Japan for several types of chronic and persistent pains, including neuropathic pain. It has been shown to potentiate the analgesic effects of some kinds of *Kampo* medicine.[21] Bushi, at a dose of 0.3 g/kg, improved cold allodynia (Fig. 3A) and significantly potentiated the effects of GJG against it (Fig. 3C). These results suggest that bushi may play a pivotal role in the ameliorating effects of GJG against cold allodynia and that a combination of GJG and bushi may contribute to potentiating the action against it. However, a further study to examine the effect of GJG eliminating bushi on cold allodynia is needed to clarify whether bushi is the active ingredient of GJG against it. Interestingly, neither GJG alone nor bushi alone at a dose of 0.3 g/kg inhibited the oxaliplatin-induced mechanical allodynia (Fig. 3B), but when co-administered, they significantly inhibited mechanical allodynia (Fig. 3D). Although the mechanisms underlying mechanical allodynia are not simple, allodynia includes both peripheral and central neuronal phenomena. As for central mechanisms, the activation of spinal microglia[26] or astrocytes[20] is known to be highly related to the pathogenesis of neuropathic pain. It has become apparent that glial cells, especially microglia and astrocytes, in the spinal cord play a substantial role in the pathogenesis of neuropathy induced by anti-cancer agents, such as paclitaxel, vincristine, and oxaliplatin.[27-29] Furthermore, we found that bushi is a strong inhibitor of astrocytic activation and revealed its anti-allodynic effect caused by inhibiting spinal astrocytes in the Seltzer mouse model.[20] However, in the present study, the activation of glial cells (changes in the localization and morphology of microglia and astrocytes) was not observed in the spinal dorsal horn of oxaliplatin-treated mice on days 5 and 19 using immunohistochemical analyses (data not shown). The reason for the failure of bushi alone to inhibit the oxaliplatin-induced mechanical allodynia is either because spinal astrocytes, the target cells of bushi, were not activated in our OIPN model or because the symptom types of OIPN are not exactly the same as those of neuropathic pain. Bushi has a variety of other pharmacological activities, including a thermal effect, an increase in blood flow, and the inhibition of platelet aggregation,[30-32] thus, we do not exclude the possibility that bushi may potentiate the therapeutic effects of GJG via these pharmacological activities.

In conclusion, we demonstrated that GJG prevented acute and cumulative neuropathy using an OIPN mouse model. Furthermore, we found that bushi potentiated the ameliorating effects of GJG against OIPN. Thus, the combination of GJG with bushi can improve the symptoms of patients who do not respond adequately to monotherapy with either GJG or bushi. These findings are expected to improve treatments and support future clinical trials associated with oxaliplatin therapy.

Materials and methods

Animals

Eight-week-old male ICR mice (Japan SLC, Shizuoka, Japan) were used in the present study. They were kept under controlled temperature at 23 ± 3°C and relative humidity of 50 ± 20%, with a 12-h light/dark cycle. Animals were allowed free access to solid food and water in their home cages (4–5 mice per cage). All experimental procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals approved by the Laboratory Animal Committee of Tsumura & Co.

Drugs

Oxaliplatin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). GJG is composed of 10 herbal medicines: Rehmanniae radix (5.0 g), Achyranthis radix (3.0 g), Corni fructus (3.0 g), Moutan cortex (3.0 g), Alismatics rhizome (3.0 g), Dioscoreae rhizome (3.0 g), Plantaginis semen (3.0 g), Hoelen (3.0 g), processed Aconiti tuber (1.0 g), and Cinnamomi cortex (1.0 g). This drug was prepared as a spray-dried powder from a hot-water extract (yield 16%) and obtained from Tsumura & Co. (Tokyo). Bushi (TJ-3023), a processed *Aconiti tuber*, was also obtained from Tsumura & Co. Oxaliplatin was dissolved in 5% glucose solution. GJG and bushi were dissolved in distilled water.

Experimental schedule

To establish an OIPN mouse model, oxaliplatin (4 mg/kg body weight) or its vehicle (5% glucose solution) was injected intraperitoneally twice a week for 4 weeks (days 1, 2, 8, 9, 15, 16, 22, and 23). GJG (0.3 or 1 g/kg body weight) and bushi (0.1 or 0.3 g/kg body weight) were administered orally 5 times a week for up to 4 and 3 weeks, respectively. The acetone test and cold plate test, which are used to assess cold sensations, and the hot plate test, which is used to assess heat sensations, were performed on days 0, 3, 10, 17, and 24. The von Frey test, which is used to assess mechanical allodynia, was performed on days 0, 4, 11, 18, and 25. The neurometer test, which is used to assess the threshold of peripheral sensory neurons to electrical stimuli, was performed on days 0, 5, 12, 19, and 26.

Assessment of cold hyperalgesia and cold allodynia

Cold hyperalgesia induced by oxaliplatin was assessed using a cold plate test, which was performed according to the method described by Mizuno et al.[18] Briefly, mice were placed on a Hot/Cold Plate Analgesia Meter (MK-350HC, Muromachi Kikai Co., Ltd, Tokyo, Japan), the temperature of which was kept at 4°C. The latency of withdrawal responses, such as the elevation and licking of each hind paw, during 150 s were recorded. Cold allodynia induced by oxaliplatin was assessed using the acetone test. Approximately 50 μl acetone (Wako Pure Chemical Ltd., Osaka, Japan) was sprayed onto the plantar skin of the right hind paw, and the time spent in the elevation and licking of the stimulated hind paw during 60 s was measured.

Assessment of thermal hyperalgesia

The hot plate test was performed to assess thermal hyperalgesia. Mice were placed on a Hot/Cold Plate Analgesia Meter, the temperature of which was kept at 50°C. A cut-off time of 45 s was set to prevent tissue damage. The latency of withdrawal responses, such as the licking and flinching of each hind paw, was recorded.

Assessment of mechanical allodynia

The von Frey test, which was used to assess mechanical allodynia, was performed according to the method described by Shibata et al.[20] Briefly, a 0.16 g von Frey filament was applied to the plantar surface of the right hind paw. The paw withdrawal in response to the tactile stimulus was scored as follows: 0, no response; 1, a withdrawal response away from the stimulus with slight flinching and/or licking; and 2, an intense withdrawal response away from the stimulus with brisk flinching and/or licking. One trial involved 10 applications of the filament, each of which was scored as 0, 1, or 2. The trial was evaluated based on a total score of 0–20 at the culmination of the test (% of maximum score).

Measurement of current perception threshold

The neurometer test, which was used to assess the thresholds of peripheral sensory neurons, was performed according to the method described by Matsumoto et al.[22] Briefly, electrodes (Neurotron Inc., Baltimore, MD) were attached to the right planter surfaces of the hind paw. Transcutaneous neuronal stimuli (sine-wave pulses of 5, 250, or 2000 Hz for activation of the C-, Aδ-, or Aβ-fibers, respectively) were applied using a Neurometer® CPT®/C (Neurotron Inc.). The minimum intensity (μA) at which each mouse showed a withdrawal response of the stimulated hind paw was defined as the current threshold.

Statistical analysis

All results are expressed as the mean ± standard error of the mean. All statistical analyses were performed using StatLight2000 software (Yukms Co., Ltd, Tokyo, Japan). All behavioral data, except for that of the von Frey test, were analyzed using one-way analysis of variance (ANOVA) with post hoc multiple comparison using Tukey's test. The data for the von Frey test were analyzed using ANOVA performed with Scheffé's multiple comparison tests. Differences resulting in P values of <0.05 were considered statistically significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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