Introduction

Oxaliplatin, a third-generation platinum drug, is widely used with 5-fluorouracil/leucovorin (5-FU/LV) for treatment of advanced/recurrent colorectal cancer (1–3). Peripheral neuropathy occurs as an adverse effect of oxaliplatin with extraordinary frequency and is the most common dose-limiting factor for oxaliplatin treatment (4–6). Peripheral neuropathy observed with oxaliplatin treatment can be classified as two distinct symptoms: 1) acute peripheral neuropathy, which occurs during or immediately after infusion of oxaliplatin and remains for a few days, and 2) dose-limiting, cumulative sensory neuropathy. Acute neuropathy is observed in approximately 90% of patients after administration of oxaliplatin, and it presents as paresthesia and dysesthesia in the extremities and perioral region, with jaw tightness. These characteristic responses to oxaliplatin may be triggered or potentiated by exposure to cold (7, 8). In most cases, the acute neuropathy attributed to oxaliplatin occurs at
every treatment, although it is ameliorated after cessation of the drug. Prevention and improvement of the peripheral neuropathy related to oxaliplatin therapy is very important to ameliorate the patient’s quality of life and to encourage continuation of the treatment. However, at present, there are no effective treatments or preventive measures for oxaliplatin-associated neuropathy. As treatment for oxaliplatin-induced peripheral neuropathy, Gamelin et al. (11) reported that infusion of calcium gluconate and magnesium sulfate (Ca/Mg), which chelates oxalate, a metabolite of oxaliplatin, before and after oxaliplatin treatment might reduce the incidence and intensity of acute neuropathy and delay cumulative neuropathy in clinical trials. This medication is currently expected to treat oxaliplatin-induced neuropathy, but the effectiveness of Ca/Mg infusions against oxaliplatin-induced acute neuropathy remains controversial in clinical practice (12, 13).

Goshajinkigan (GJG), a traditional Japanese (kampo) medicine, is composed of 10 herbal medicines in fixed proportions. GJG is widely used to treat rhizositis or numbness in the extremities, low back pain, melosalgia, dysuria, and diabetic neuropathy (14–16). Recently, Kono et al. reported that GJG prevented oxaliplatin-induced neurotoxicity in a placebo-controlled double-blind randomized phase II study (the GONE Study) (17). The effect of GJG against cumulative oxaliplatin-induced neuropathy was indicated, but the efficacy of GJG against acute neuropathy remains unproved. On the other hand, a basic study of a rodent model of oxaliplatin-induced neuropathy also demonstrated that GJG ameliorated oxaliplatin-induced neuropathy and notably relieved the cold hypersensitivity caused by oxaliplatin (18). However, the mechanism of how GJG prevents oxaliplatin-induced acute neuropathy remains uncertain.

Transient receptor potential (TRP) channels, Ca2+-permeable nonselective cation channels, are suggested to serve as thermal, chemical, and mechanical sensors (19, 20). Among the TRP channels, TRPV1 responds to noxious heat (21–23), whereas TRPA1 and TRPM8 respond to noxious cold (21, 24, 25) and innocuous cooling (26, 27), respectively. These TRP channels are expressed in sensory neurons of dorsal root ganglia (DRG) and trigeminal ganglia (TG), and they are primary detectors of various environmental insults (28, 29). Recently, accumulating evidence indicates that these TRP channels are responsible for chemotherapy-induced neuropathy. In particular, TRPA1 and TRPM8 have been reported to be involved in acute oxaliplatin-induced neuropathy (29–36).

In this study, we examined the effect of GJG on acute neuropathy using an oxaliplatin-induced neuropathy rat model. Then, to elucidate the mechanism of the amelio-
Cold stimulation
Oxaliplatin-induced cold hyperalgesia was assessed using a cold plate according to the method described by Sakurai et al (37). Briefly, rats were placed on a hot/cold plate analgesia meter (MK-350HC; Muromachi Kikai Co., Ltd., Tokyo) with the temperature of the plate at 4°C. The latency and the number of withdrawal responses such as elevating and licking of a hind paw during 150 s were recorded.

Cold allodynia induced by oxaliplatin was assessed by measuring acute nocifensive responses to acetone evaporation–evoked cooling (acetone test). The animals were habituated to a cage with a wire mesh floor for more than 30 min prior to testing. Acetone (250 μl; Wako Pure Chemical Ltd., Osaka) was sprayed onto the plantar skin of the right hind paw with a micro-syringe, and the time spent in elevating and licking the stimulated hind paw was measured for 60 s. The acetone test was performed twice at a 15-min interval, and the average of withdrawal response time was calculated.

Allyl isothiocyanate (AITC)-induced nocifensive behavioral test
AITC, a TRPA1 agonist, was used to activate peripheral TRPA1 and elicit nocifensive behaviors in rats (38). The AITC-induced nocifensive behavioral test was performed on day 3. Following habituation to the testing apparatus, 100 μl AITC (1% in saline) was injected into the plantar surface of the right hind paw, and the total number of nocifensive behaviors (lifting, licking, and flinching of the injected paw) was recorded for 5 min. In the control group, the hind paw was injected with an equal volume of saline.

Menthol-induced nocifensive behavioral test
Menthol, a TRPM8/TRPA1 agonist, evokes a cooling sensation when applied to the skin (36, 39). To assess the effect of menthol on nocifensive behaviors in an oxaliplatin-induced acute neuropathy model, a menthol test was carried out on day 3. Following habituation to the testing apparatus, 250 μl of (l)-menthol (100 mM in 90% DMSO and 10% PBS) or its vehicle was applied to the plantar surface of the right hind paw, and the duration of menthol-induced nocifensive behaviors (licking and flinching of the stimulated hind paw) was measured for 5 min. This test was performed twice with a 15-min interval, and the average of response time was calculated.

Capsaicin-induced eye wiping test
Capsaicin is known to be a TRPV1 agonist. Especially when applied to the eyes, capsaicin evokes eye wiping movements through TRPV1 activation (40). On day 3, rats were placed in a plastic cage to habituate themselves for more than 15 min and 50 μl of capsaicin (0.1%, dissolved in 10% ethanol, 10% Tween80, and 80% saline) or vehicle alone was dropped into the right eye of the rats. The number of eye wiping movements was recorded for 2 min.

Real-time polymerase chain reaction (PCR)
Lumbar L4-L6 DRG were isolated from each group on day 3 of oxaliplatin treatment. Total RNA was isolated from tissues using a tissue homogenizer (Micro Smash TM MS-100R; Tomy Seiko, Ltd., Tokyo), QIAzol lysis reagent, and an RNaseasy mini kit (Qiagen, Germany), according to the manufacturer’s instructions. The amounts of total RNA in each sample were quantified on a Nanodrop ND-1000 spectrophotometer (V3.0.1; LMS Co., Ltd., Tokyo). Reverse transcription of 1 μg total RNA was carried out using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s directions. Two sets of primer-probe were designed by the Primer Express Software (Applied Biosystems). The primers and TaqMan MGB probes for rat transient receptor potential cation channel, subfamily V, member 1 (TRPV1, Rn00583117_m1, FAM), transient receptor potential cation channel, subfamily A, member 1 (TRPA1, Rn01473803_m1, FAM), transient receptor potential cation channel, subfamily M, member 8 (TRPM8, Rn00592665_m1, FAM), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Rn01775763_g1, FAM) were purchased from Applied Biosystems. Quantitative real-time PCR was performed using a 7900HT Fast Real Time PCR system (Applied Biosystems). Experiments were performed in triplicate. The fold change of each target was calculated relative to the internal control GAPDH mRNA levels.

Statistical analyses
All results are expressed as the means ± S.E.M. All statistical analyses were performed with StatLight2000 (Yukms Co., Ltd., Tokyo). The statistical significance of the difference between control and oxaliplatin-treated groups was calculated using Student’s t-test. The behavioral data for GJG and calcium gluconate/magnesium sulfate tests and real time-PCR data were analyzed by a one-way analysis of variance and post hoc multiple comparison using Dunnett’s test. A difference was considered significant at P < 0.05.

Results
Goshajinkigan or Ca/Mg injections prevented oxaliplatin-induced cold hypersensitivity in rats
Administration of oxaliplatin (4 mg/kg, i.p., once
daily for 2 days) significantly increased the duration of withdrawal responses to cold stimulation by acetone (Fig. 1) and the cold plate (Fig. 2). In the acetone test, GJG (0.3 or 1 g/kg, p.o., once daily for 2 days) prevented cold allodynia induced by oxaliplatin in a dose-dependent manner (Fig. 1A). Furthermore, GJG also inhibited the oxaliplatin-induced reduction of latency for cold simulation in the cold plate test (Fig. 2). Similarly, i.p. injections of Ca/Mg (0.5 mmol/kg), 30 min before administration of oxaliplatin suppressed the oxaliplatin-induced cold allodynia in the acetone test (Fig. 1A). On the other hand, there were no significant differences in the latency of withdrawal response to cold stimulation between Ca/Mg [0.08 mmol Ca + 0.16 mmol Mg / kg, a concentration that corresponds to that of GJG (1 g/kg) solution, p.o., once daily for 2 days] on the acetone test in oxaliplatin-treated groups (Fig. 1B). Combination treatment with both Ca/Mg and GJG potentiated the effects of 0.3 g/kg GJG treatment without counteracting the effect of each on cold hypersensitivity induced by oxaliplatin (Fig. 1A).

**Oxaliplatin potentiated AITC and menthol-evoked nocifensive response in rat**

In oxaliplatin-treated rats, intraplantar injection of 1% AITC, a selective TRPA1 agonist, potentiated the nocifensive behaviors (duration of licking and flinching of the injected hind paw) compared to that of vehicle-treated rats (Fig. 3A). Moreover, application of 100 mM (l)-menthol, a TRPA1 and TRPM8 agonist, to the plantar surface of the hind paw also enhanced nocifensive behaviors in the oxaliplatin-treated group (Fig. 3B), whereas no significant difference in the number of eye-wiping movements evoked by capsaicin, a TRPV1 agonist, was seen between groups (Fig. 3C). As shown in
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Fig. 3: D–F, TRPA1 and TRPM8 mRNA expression levels were significantly increased in DRG on day 3 after oxaliplatin treatment. However, the expression level of TRPV1 mRNA was not altered by oxaliplatin.

Goshajinkigan suppressed AITC- and menthol-evoked nocifensive response in oxaliplatin-treated rats

As shown in Fig. 3, A and B, co-administration of GJG (0.3 or 1 g/kg, p.o., once daily for 2 days) suppressed the duration of AITC-evoked withdrawal responses enhanced by oxaliplatin in a dose-dependent manner. GJG also prevented menthol-evoked withdrawal responses potentiated by oxaliplatin, whereas administration of GJG did not produce a significant increase in the expression levels of TRPA1 or TRPM8 mRNA compared with control groups (Fig. 3: D–F).

Discussion

In the present study, we demonstrated that GJG was as efficacious as injections of Ca/Mg in ameliorating oxaliplatin-induced acute neuropathy using a rat model. Further, we found that GJG inhibited the menthol- and AITC-evoked withdrawal responses enhanced by oxaliplatin treatment. These results suggest that TRPM8 and/or TRPV1 are involved in the preventive effects of GJG against cold hypersensitivity induced by oxaliplatin.

Oxaliplatin, a third-generation platinum drug, is widely
used as a standard chemotherapy for advanced/recurrent colorectal cancer (1–3). Peripheral neuropathy observed with oxaliplatin can be classified as acute or chronic neuropathy (the cumulative sensory neuropathy) according to the different symptoms (4–6). The acute neuropathy occurs frequently during or immediately after infusion of oxaliplatin and presents as paresthesia and dysesthesia in the extremities and perioral region with jaw tightness that is aggravated by exposure to cold (7, 8). On the other hand, the chronic neuropathy is characterized by sensory ataxia, functional impairment, jaw pain, eye pain, ptosis, leg cramps, and visual and voice changes (4–6); and it resembles that of other platinum-based agents such as cisplatin (5, 9, 10). Therefore, the chronic neuropathy can be dose-limiting and may require discontinuation of treatment. Although the acute neuropathy is not a reason to reduce the dose or discontinue the medication, the use of drugs that prevent the symptoms has an important clinical implication because it improves the patient’s quality of life during continued treatment. At present, there is no established effective treatment or preventive measure for peripheral neuropathy associated with oxaliplatin therapy. Basically, the current approach for oxaliplatin-induced peripheral neuropathy is the “stop and go” therapy of dose reduction and a change to other drugs if symptoms worsen. As medication for oxaliplatin-induced peripheral neuropathy, Gamelin et al. (11) reported that infusion of Ca/Mg, working as oxalate chelators, before and after oxaliplatin treatment might reduce the incidence and intensity of acute neuropathy and delay the cumulative neuropathy in clinical trials. Indeed, as shown in Fig. 1A, injection of Ca/Mg before administration of oxaliplatin prevented the oxaliplatin-induced cold hypersensitivity in our rodent model.

GJG, a traditional Japanese herbal medicine, is widely used to treat rhigos or numbness in the extremities, low back pain, melosalgia, dysuria, and diabetic neuropathy (14–16). Recently, Kono et al. reported that GJG exhibited a preventive effect against oxaliplatin-induced peripheral neurotoxicity in a placebo-controlled double-blind randomized phase II study (17), and it has been attracting attention as a therapeutic drug for chemotherapy-induced peripheral neuropathy. Furthermore, in a rodent model, administration of GJG improved the peripheral neuropathy induced by oxaliplatin treatment, and it notably relieved the cold hypersensitivity caused by oxaliplatin (18). However, the mechanism by which GJG ameliorates the oxaliplatin-induced acute peripheral neuropathy remains to be defined. Our behavioral study on the oxaliplatin-induced neuropathy rat model demonstrated that administration of GJG significantly inhibited cold hypersensitivity induced by oxaliplatin in a dose-dependent manner (Figs. 1, 2). Especially, 1 g/kg GJG showed equivalent efficacy to infusions of Ca/Mg for cold hypersensitivity. These results suggest that the mechanism of action of GJG on oxaliplatin-induced acute peripheral neuropathy is likely to have a mechanism similar to that of Ca/Mg infusions. However, according to the description of the ingredients of GJG described in the information sheet, the amounts of calcium and magnesium in the 1-g extract powder of GJG used in our behavioral tests are about 10% of the applicable dose of Ca/Mg (0.5 mmol/kg body weight). Therefore, we examined the effect of Ca/Mg (0.08 mmol Ca + 0.16 mmol Mg/kg), a concentration corresponding to that of GJG (1 g/kg) solution, on acute neuropathy. As shown in Fig. 1B, the administration of Ca/Mg had no effect on cold allodynia induced by oxaliplatin. Thus, the mechanism of action of GJG on oxaliplatin-induced acute neuropathy might not be the same as that of Ca/Mg infusions.

A medication containing Ca/Mg is currently expected to treat oxaliplatin-induced neuropathy, but the effectiveness of infusions of Ca/Mg against oxaliplatin-induced acute neuropathy in clinical practice remains controversial (12, 13). Therefore, we investigated whether a combination of Ca/Mg and GJG produces additional effects on oxaliplatin-induced acute peripheral neuropathy. As shown in Fig. 1A, treatment with a combination of Ca/Mg and GJG potentiated the effects of 0.3 g/kg GJG treatment without counteracting the beneficial effect of each on the cold hypsersensitivity induced by oxaliplatin. Thus, combination therapy with Ca/Mg and GJG may be expected to improve the symptoms of patients who did not respond adequately to monotherapy.

TRP channels, Ca²⁺-permeable nonselective cation channels, have been proposed to serve as thermal, chemical, and mechanical sensors (19, 20). These TRP channels are expressed in nerve endings of sensory neurons in the peripheral nervous system, and they are the primary detector of various environmental insults (28, 29). Recently, accumulating evidence indicated that these thermosensitive TRP channels are responsible for the chemotherapy-induced peripheral neuropathy (29–36). In particular, TRPA1 and TRPM8, which respond to noxious cold stimulation (21, 24, 25), and innocuous cooling (26, 27), respectively, have been reported to be involved in oxaliplatin-induced cold hypersensitivity (31–36). Several studies with TRPA1-null mice and a TRPA1 antagonist improved oxaliplatin-induced cold allodynia (31, 34, 35). Likewise, deletion of the TRPM8 gene and treatment with capsazepine, a TRPM8 antagonist, attenuated cold hypersensitivity in oxaliplatin-induced neuropathic pain models (32, 33, 36). According to our real-time PCR results, administra-
tion of oxaliplatin increased the expression levels of TRPA1 and TRPM8 mRNA in DRG on day 3 (Fig. 3: D, E). Furthermore, behavioral tests revealed that intra-plantar injection of AITC significantly potentiated the nocifensive behaviors evoked by AITC, compared to that in vehicle-treated rats (Fig. 3A). Moreover, the application of menthol on the plantar surface of the hind paw also enhanced the nocifensive behaviors evoked by menthol in the oxaliplatin-treated group (Fig. 3B). These results are consistent with previous studies and suggested that oxaliplatin may cause a functional alteration in TRPA1 and/or TRPM8 activity. Interestingly, oxaliplatin did not alter the expression levels of TRPV1 mRNA in the DRG on day 3 (Fig. 3F). Additionally, there were no significant differences between control and oxaliplatin-treated groups in eye wiping movement induced by capsaicin, a TRPV1 agonist (Fig. 3C). We also confirmed that oxaliplatin treatment did not change the sensation of heat in a hot plate test (data not shown). Although it is not known exactly how oxaliplatin selectively alters TRPA1 and TRPM8 functions, the characteristic symptoms of acute neuropathy might be developed by this phenomenon.

According to our results, administration of GJG prevented the increase of mRNA expression levels of TRPA1 and TRPM8 induced by oxaliplatin. In behavioral tests, GJG significantly and definitely suppressed AITC- or menthol-evoked nocifensive responses. These results suggest that GJG might prevent oxaliplatin-induced cold hypersensitivity via inhibition of mRNA expression levels of TRPA1 and/or TRPM8 in the DRG of oxaliplatin-treated rats. Kawashiri et al. demonstrated that oxaliplatin treatment increased the expression level of TRPM8 mRNA through activation of L-type Ca<sup>2+</sup> channels and induction of nuclear factor of activated T-cell (NFAT) translocation into the nucleus of DRG cells (33). Although the molecular targets of GJG remain unknown, GJG might ameliorate the cold hypersensitivity induced by oxaliplatin by controlling neuronal excitability. In the preliminary study, many ingredients that were reported to have various pharmacological activities (e.g., neuroprotective, anti-oxidative, anti-convulsant, anti-inflammatory, or analgesic) were detected in the plasma of GJG-treated rats (unpublished data). Further studies are needed to clarify the detailed mechanism of action of GJG on oxaliplatin-induced acute neuropathy and identify the main active component in GJG.

In this study, we demonstrated that GJG ameliorated oxaliplatin-induced acute peripheral neuropathy by suppressing the functional alteration of TRP channels, especially TRPA1 and TRPM8, which work as sensors of cold. These findings are expected to improve treatments using oxaliplatin and support for future clinical trials associated with oxaliplatin therapy.

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**Conflicts of Interest**


**References**


