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Report

Effects of Shakuyakukanzoto on Decreased Grip Strength and Blood Flow, and Muscle Atrophy in Mice Treated with Paclitaxel

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Paclitaxel (PTX) is an anticancer drug that induces peripheral neuropathy-associated muscle weakness. However, only a few effective therapeutic agents are currently available. Shakuyakukanzoto (SKT) is a traditional kampo medicine that consists of two herbal medicines, Paeoniae radix (PR) and Glycyrrhizae radix (GR). SKT is used to treat muscle-related pain. Recently, we found that mice treated with PTX exhibited decreased grip strength. In the present study, we demonstrated that SKT attenuated the decrease in grip strength in mice treated with PTX. PTX was injected intraperitoneally once daily, every other day, a total of four times in mice. Dried water extract (WE)-SKT, WE-PR, and WE-GR were orally administered once a daily. PTX induced a reduction in grip strength. WE-SKT, but not WE-PR or WE-GR, inhibited the decrease in grip strength in PTX-treated mice. PTX decreased peripheral blood flow in mice. WE-SKT and WE-PR, but not WE-GR, inhibited the decrease in peripheral blood flow in PTX-treated mice. Histochemical staining showed that PTX induced skeletal muscle atrophy, whereas WE-SKT, but not WE-PR and WE-GR, inhibited atrophy. PTX reduced proliferation and F-actin formation in C2C12 cells. WE-SKT did not inhibit the above action of PTX in C2C12 cells. These results show that the direct action of PTX on skeletal muscles and PTX-induced muscle atrophy may be involved in the decrease in grip strength and peripheral blood flow. It is suggested that the improvement of these things by SKT contributes the inhibitory action of SKT on PTX-induced muscle weakness.

Key words Shakuyakukanzoto, Paclitaxel, Grip strength, Skeletal muscle, Peripheral neuropathy

INTRODUCTION

Paclitaxel (PTX) is a chemotherapeutic medication used to treat ovarian cancer, breast cancer, and non-small cell lung cancer.[1-3] PTX induces peripheral neuropathy (e.g., pain, allodynia, numbness, and muscle weakness) that is difficult to control with existing analgesics and adjuvant analgesics.[4-7] Therefore, therapeutic medicines for peripheral neuropathy induced by chemotherapy are needed.

Shakuyakukanzoto (SKT) is a traditional herbal medicine that consists of two components, Paeoniae radix (PR) and Glycyrrhizae radix (GR), and is used to treat muscle pain and spasms, joint pain, and numbness in human patients.[8,9] SKT attenuates PTX-induced mechanical allodynia in mice.[10-12] We showed that the components of PR, but not GR, are important for the suppression of mechanical allodynia by SKT.[10] Paeoniflorin, a major component of PR, inhibits PTX-induced mechanical allodynia.[13] Patients treated with PTX develop muscle weakness.[4] In mice treated with PTX, we reported a decrease in grip strength due to muscle weakness.[10] In addition, PTX decreases peripheral blood flow in mice.[14,15] In rats, PTX also decreases sciatic nerve blood flow.[16] Changes in blood flow caused by PTX may contribute to numbness, which is a symptom of peripheral neuropathy. Paeoniflorin improves blood flow.[17,18] Therefore, in the present study, we demonstrated that SKT inhibited the decreased in grip strength and peripheral blood flow.

MATERIALS AND METHODS

Animals Male C57BL/6NCr mice (6 weeks old; Japan SLC, Ltd., Hamamatsu, Japan) were used. They were housed in a room with controlled temperature (21–23°C), humidity (45%–65%), and a 12 h light/dark cycle (lights on from 8:00 am to 8:00 pm). They were provided *ad libitum* access to food and water. All experimental animal procedures were approved by the Committee for Animal Experiments of Kinjo Gakuin University (No. 193, 260).

Drugs PTX (Tokyo Chemical Industry Co., Ltd., Tokyo) was dissolved in physiological saline containing 10% Cremophor EL® (Sigma-Aldrich) and 10% ethanol and administered intraperitoneally (i.p.) once daily 4 times every other day (Day

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0, 2, 4, 6) at a volume of 0.1 ml/10g of body weight (final dosage of 8 mg/kg/time).[10,19]

The dried water extract (WE)-SKT (Lot. No. 2200068010, 2021), WE-PR (Lot. No. 2191001010, 2021), and WE-GR (Lot. No. 2191013010, 2021) were obtained from Tsumura & Co., Ltd. (Tokyo). These dried extracts were dissolved in 5% gum arabic (FUJIFILM Wako Pure Chemicals Co., Osaka) and administered orally once a day after the behavioral evaluation. When PTX (or the vehicle) was injected intraperitoneally, WE-SKT, WE-PR, WE-GR, or their vehicle was administered orally 1 h after injection of PTX (or the vehicle) at a volume of 0.1 ml/10 g of body weight.

Behavioral Evaluation Grip strength was evaluated using a digital grip strength meter (GPM-101B/V; Melquest Ltd., Toyama). The evaluation was performed three times, and the average value was used as the value for that individual.

Measurement of Peripheral Blood Flow After behavioral evaluation on the day following the last administration, under combined anesthesia (0.03 mg/ml, medetomidine hydrochloride [Dorbene®, Kyoritsuseiyaku Corp., Tokyo], midazolam [0.4 mg/ml Dormicum®, Maruishi Pharmaceutical Co., Ltd., Osaka] and butorphanol [0.5 mg/ml, Vetorphale®, Meiji Seika Pharma Co., Ltd., Tokyo]: 0.1 ml/10g body weight, i.p.), the blood flow rate was measured using a laser Doppler blood flow meter with a contact probe (ALF21, ADVANCE Co., Ltd., Tokyo).

Hematoxylin and Eosin Staining After behavioral evaluation and blood flow measurement on the day following the last administration, the mice were sacrificed under mixed anesthesia, and transcardially perfused with phosphate-buffered saline (pH 7.4). The hind legs were removed and fixed with 4% paraformaldehyde (FUJIFILM Wako Pure Chemicals Co.), and calf muscles isolated from the hind legs were treated with 30% sucrose. One week later, the muscles were embedded in OCT compound and stored at -80°C until use. Frozen skin sections were stained with the Hematoxylin and Eosin (HE). After dehydration with ethanol and clearing with xylene, the sections were mounted with Canada balsam and observed under a light microscope (BX43, Olympus, Tokyo).

Cell Culture Mouse C2C12 myoblasts were purchased from American Type Culture Collection (ATCC CRL-1772, Manassas, VA, USA). Cells were cultured and maintained in tissue culture dish (TPP, Trasadingen, Switzerland) in growth medium containing high glucose Dulbecco's modified Eagle medium (DMEM) (FUJIFILM Wako Pure Chemicals Co.) supplemented with 10% fetal bovine serum (FBS) (Hyclone™, Cytiva, Marlborough, MA, USA) and 1% penicillin–streptomycin (FUJIFILM Wako Pure Chemicals Co.) in an incubator at 37°C under a humidified atmosphere of 5% CO_2. Cells were grown to confluence, transferred to differentiation medium containing 2% horse serum (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA), and incubated for 7 days.

Immunocytochemistry The cells were fixed with Mildform® 10N (FUJIFILM Wako Pure Chemicals Co.). Cells were double stained with Alexa-Fluro™ 568 phalloidin (A12380, Invitrogen, Waltham, MA, USA) and DAPI (62248, Thermo Fisher Scientific), and examined using a confocal microscope (Fluoview FV1000; Olympus) mounted on an Olympus IX81 epifluorescence microscope with a UPlanSApo 60×/1.35 oil immersion objective lens (Olympus).

Statistical Analyses All data are presented as the mean and standard error of the mean. Statistical significance was determined using one-way analysis of variance followed by a post hoc Holm-Šidák test. Statistical significance was set at $p < 0.05$.

RESULTS

The dosages of PTX, WE-SKT, WE-PR and WE-GR used in this study were in accordance with those used in our previous report.[10] The intraperitoneal injection of PTX once daily four times every other day (days 0, 2, 4, and 6) induces

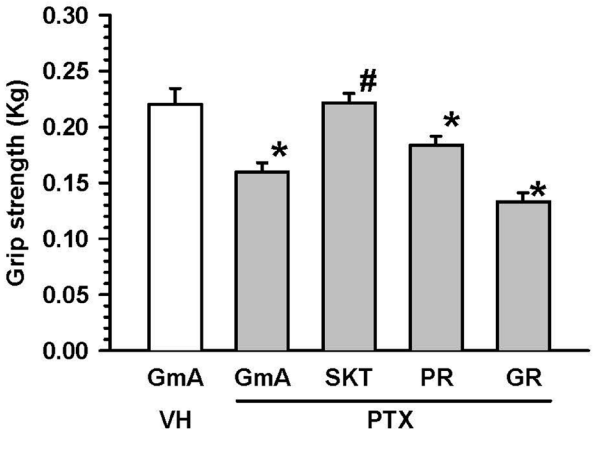

Fig. 1. Effects of Water Extract (WE) of Shakuyakukanzoto (SKT), Paeoniae Radix (PR) or Glycyrrhizae Radix (GR) on the Paclitaxel (PTX)-induced Reduction in Grip Strength in Mice.

On days 0, 2, 4 and 6, mice were intraperitoneally injected with PTX (8 mg/kg) or vehicle (VH). Gum Arabic (GmA, 5%), SKT (1 g/kg), PR (1 g/kg) or GR (1 g/kg) was administered orally once a day. When PTX was injected intraperitoneally, GmA or WE of herbal medicines (SKT, PR, GR) was administered orally 1 h after PTX injection. Grip strength was measured on the day after the last administration. The data represent the mean ± SEM. *p<0.05 vs. VH+GmA, #p<0.05 vs. PTX+GmA (n = 6)

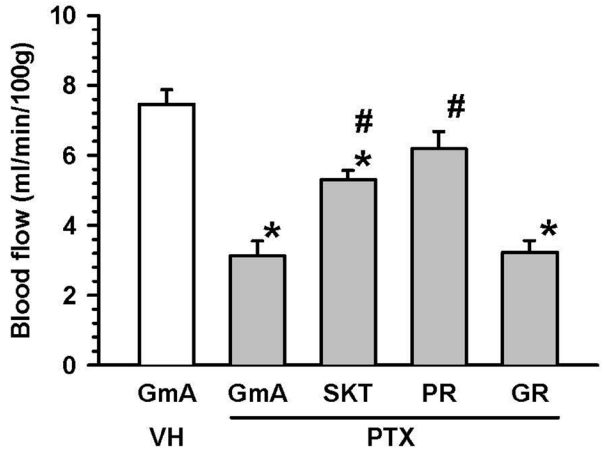

Fig. 2. Effects of Water Extract (WE) of Shakuyakukanzoto (SKT), Paeoniae Radix (PR) or Glycyrrhizae Radix (GR) on the Paclitaxel (PTX)-induced Reduction in the Peripheral Blood Flow in Mice.

On days 0, 2, 4 and 6, mice were intraperitoneally injected with PTX (8 mg/kg) or vehicle (VH). Gum Arabic (GmA, 5%), SKT (1 g/kg), PR (1 g/kg) or GR (1 g/kg) was administered orally once a day. When PTX was injected intraperitoneally, GmA or WE of herbal medicines (SKT, PR, GR) was administered orally 1 h after the injection of PTX. Peripheral blood flow was measured on the day after the last administration. The data represent the with mean ± SEM. *p<0.05 vs. VH+GmA, #p<0.05 vs. PTX+GmA (n = 6)

the reduction of grip strength from day 2 after the first dose and the reduction is continued until at least day 14.[10] Repeated oral administration of WE-SKT significantly inhibited the PTX-induced reduction in grip strength relative to the oral VH administered group (Fig. 1). However, WE-PR and WE-GR did not affect the reduction in the grip strength (Fig. 1).

PTX caused a reduction in peripheral blood flow (Fig. 2). Repeated oral administration of WE-SKT significantly inhibited PTX-induced reduction in peripheral blood flow relative to the oral VH-administered group (Fig. 2). In addition, oral WE-PR inhibited this reduction (Fig. 2). However, the WE-GR treatment did not affect this reduction (Fig. 2).

To determine the effect of each drug treatment on muscle tissue, cross-sectional area and muscle fiber structure were compared on HE images. PTX caused atrophy of the calf muscles (Fig. 3A) and abnormality in the Z-line in calf muscle fiber (Fig. 3B, yellow arrowhead). Repeated oral administration of WE-SKT prevented atrophy of the calf muscle and Z-line degeneration, but WE-PR and WE-GR did not (Fig. 3).

Next, the drug effects on muscle fusion and differentiation were analyzed in C2C12. In C2C12 cells, myogenic fusion and differentiation and myofiber development can be monitored by changing fluids from growth medium to differentiation medium (Fig. 4A). PTX not only inhibited muscle fusion (Fig. 4A) and myofiber development (Fig. 4B), but also caused abnormal nuclear structure (Fig. 4B). Addition of SKT, PR and GR in the medium did not improve PTX-induced muscle fusion and differentiation and myofiber development (Fig. 5).

DISCUSSION

Patients treated with PTX develop muscle weakness as a result of peripheral neuropathy.[4] In clinical practice, muscle weakness is an indicator of discontinuation of anticancer drugs. Therefore, the control of anticancer-induced peripheral neuropathy is important for patients with cancer. In this study, PTX, which was administered intraperitoneally once daily four times every other day, decreased grip strength in mice. Repeated oral application of WE-SKT inhibited this decrease in grip strength. However, WE-PR and WE-GR were not affected. Our previous report supported these results).[10] These results suggest that both the PR and GR components are needed to

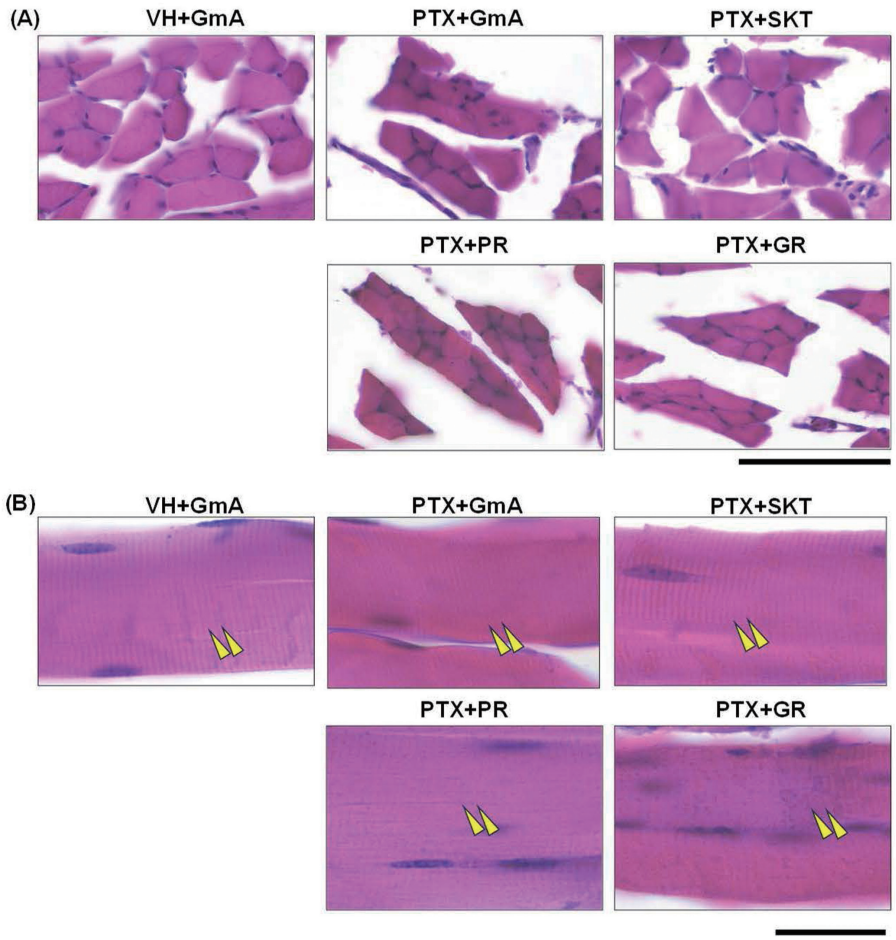

Fig. 3. Effects of Water Extract (WE) of Shakuyakukanzoto (SKT), Paeoniae Radix (PR) or Glycyrrhizae Radix (GR) on Paclitaxel (PTX)-induced Muscle Atrophy in Mice.

On days 0, 2, 4 and 6, mice were intraperitoneally injected with PTX (8 mg/kg) or vehicle (VH). Gum Arabic (GmA, 5%), SKT (1 g/kg), PR (1 g/kg) or GR (1 g/kg) was administered orally once a day. When PTX was injected intraperitoneally, GmA or WE of herbal medicines (SKT, PR, GR) was administered orally 1 h after the injection of PTX. The hind legs were isolated after the last administration. Frozen sections of the calf muscles were stained with Hematoxylin and Eosin (HE). (A) and (B) show typical examples of each cross-section and vertical section stained with HE, respectively. Arrow heads show the striated structure of the muscle. Scale bar: (A) 100 μm, (B) 20 μm.

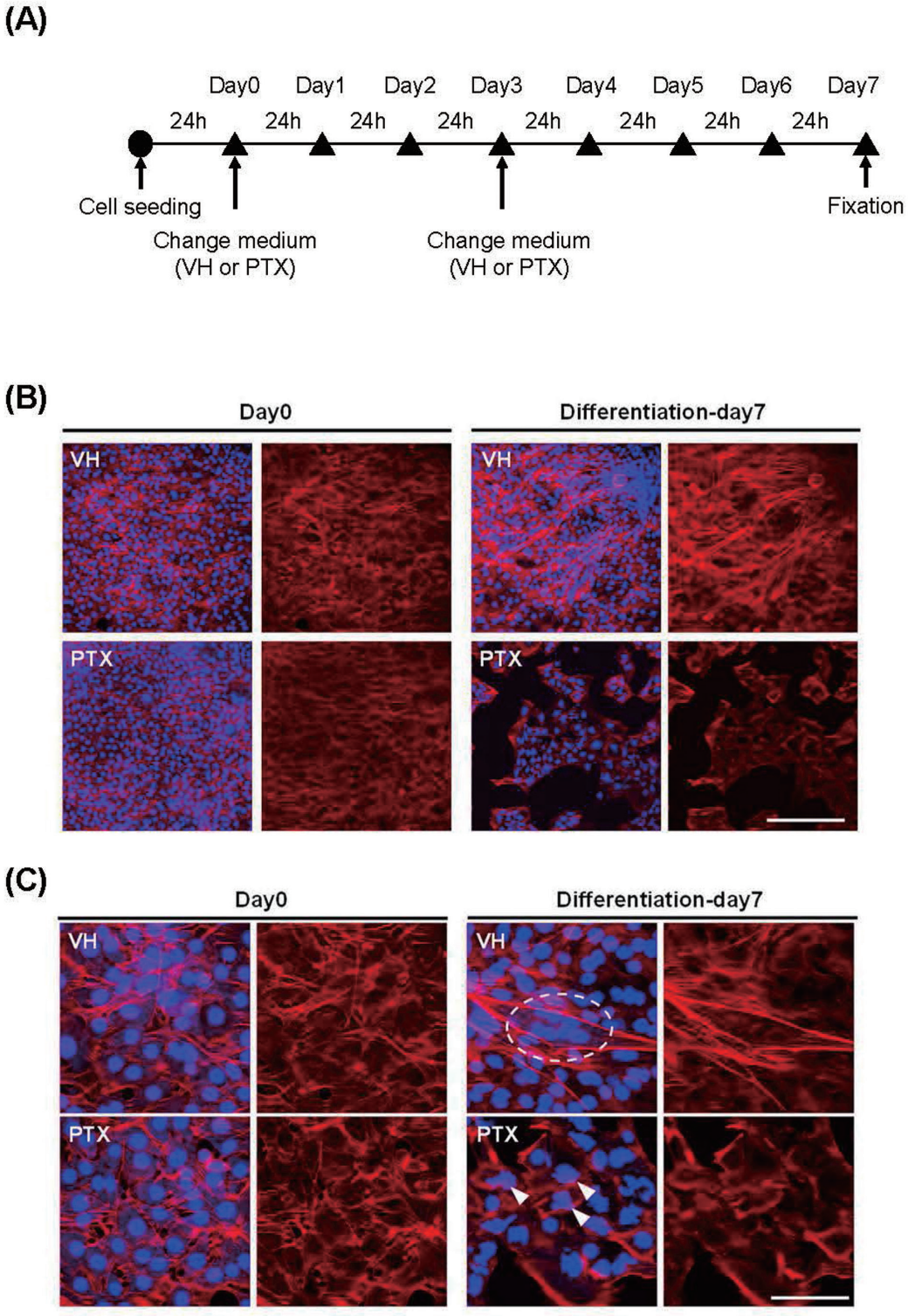

Fig. 4. Effects of Paclitaxel (PTX) on the Differentiation of Cells in the Myoblast Cell Line C2C12.

(A) The protocol of the reagent application. VH, 1% DMSO; PTX, 30 μM. (B) and the enlarged view in (C) show representative examples of the cell morphology in various treatments. Arrow heads and dotted circle show the deformed nucleus and the cell fusion for the formation of F-actin structure, respectively. F-actin and DAPI signals are shown as red and blue fluorescence, respectively. Scale bar: (B) 300 μm, (C) 100 μm.

improve the grip strength decreased by PTX.

The present study showed that PTX induced muscle atrophy and the collapse of the striated structure, which arises from the organized arrangement of actin and myosin filaments, in skeletal muscle. C2C12 cells are a well-established model for skeletal muscle cells. PTX inhibited myogenic fusion and differentiation in muscle atrophy and muscle weakness, suggesting that PTX acts directly on muscle cells. In addition, PTX reduces myosin content in C2C12 cells.[20] Reduction in myosin content leads to muscle atrophy[20,21] and muscle weakness.[22] WE-SKT,

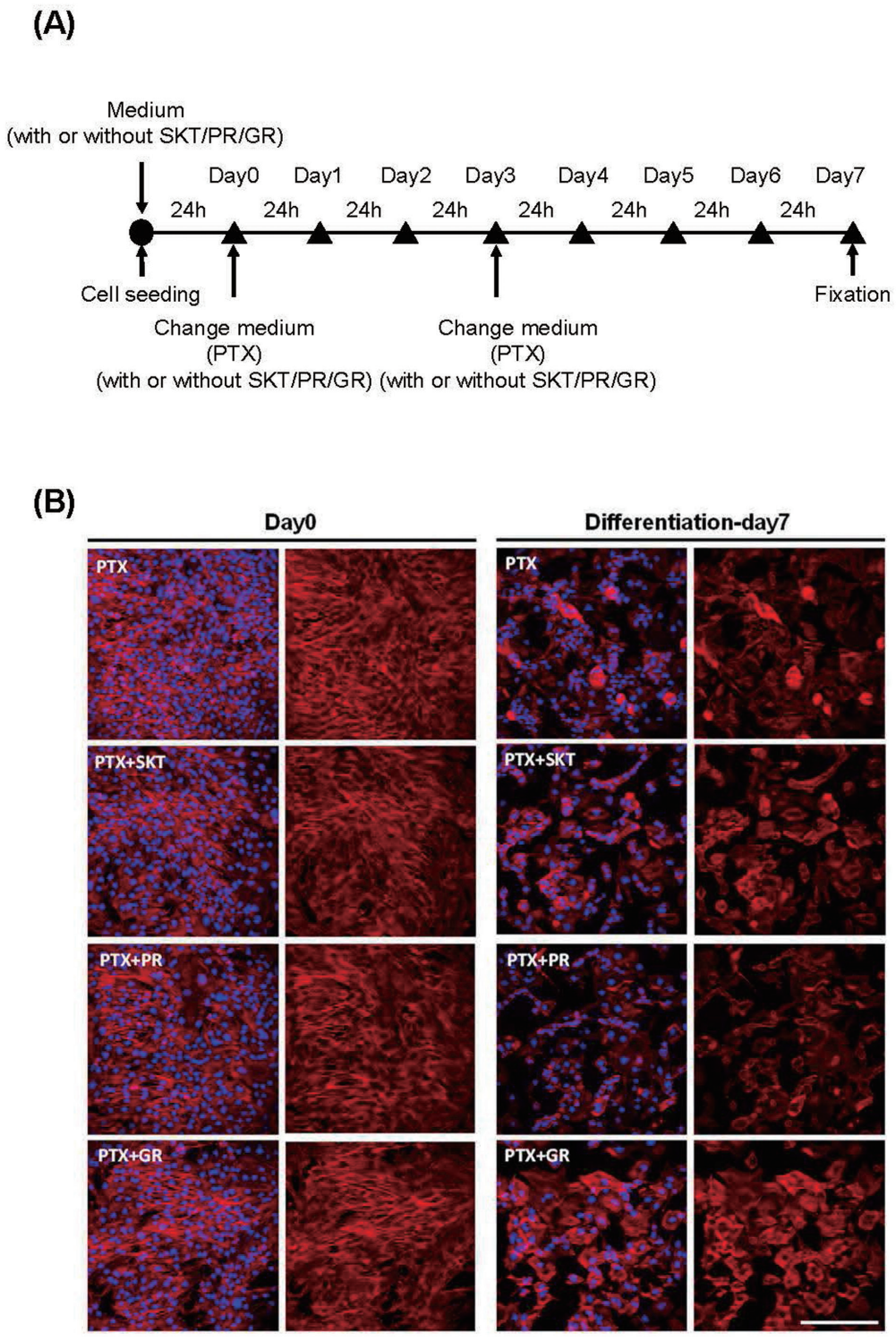

Fig. 5. Effects of Water Extract (WE) of Shakuyakukanzoto (SKT), Paeoniae Radix (PR) or Glycyrrhizae Radix (GR) on the Inhibition of the Cell Fusion for the Formation of the F-actin Structure by Paclitaxel (PTX) in Myoblasts of the C2C12 Cell Line.

(A) The protocol of the reagent application. PTX, 10 μM; SKT, PR, GR, 1 μg/mL. (B) Representative examples of the cell morphology in various treatments. F-actin and DAPI signals are shown as red and blue fluorescence, respectively. Scale bar: 300 μm.

but not WE-PR and WE-GR, inhibited PTX-induced muscle atrophy and collapse of striated structures in skeletal muscles. However, none of the WE used in this study inhibited myogenic fusion and differentiation in PTX-treated C2C12 cells. It has been suggested that the components absorbed from the intestinal tract after metabolism by glucosidase may play an important role. Pharmacokinetic profiles of the active components (e.g., albiflorin, paeoniflorin, glycycoumarin, isoliquiritigenin, glycyrrhetic acid, and glycyrrhetic acid 3-O-monoglucuronide) after the oral administration of SKT to healthy volunteers have

been reported.[23] Glycycoumarin and isoliquiritigenin have low blood concentrations and very low C*max* values. Thus, these components are thought to contribute little to the muscle atrophy or reduced blood flow. In future studies, other components should be investigated for their therapeutic effects on PTX-induced actions. Kimura *et al*., have shown that the combination of paeoniflorin and glycyrrhizin enhances the inhibition of skeletal muscle contraction.[24,25] In this study, WE-PR and WE-GR alone did not inhibit PTX-induced muscle atrophy and disorganization of myofilament in skeletal muscles. In future, we will investigate the individual effects and interactions of the active components.

PTX decreased the peripheral blood flow. However, the underlying mechanism remains unclear. As one possible mechanism, PTX-induced muscle atrophy may be involved in the decrease of peripheral blood flow. Muscle atrophy compresses the blood vessels within the active muscle, leading to a decrease in blood flow.[26,27] The prostaglandin E1 analog limaprost inhibits blood flow decrease and platelet aggregation.[28,29] Limaprost attenuates PTX-induced mechanical allodynia and blood flow decrease,[15] suggesting that control of peripheral blood flow is important for improving PTX-induced peripheral neuropathy. WE-SKT and WE-PR, but not WE-GR, inhibited PTX-induced decrease in blood flow. Paeoniflorin, a major active component of PR, improves the blood flow.[17,18] Paeoniflorin promotes the expression of eNOS/NOS3.[30] Therefore, paeoniflorin may contribute to the increased peripheral blood flow. Another possibility is that muscle atrophy may damage the blood vessels. Paeoniflorin promotes angiogenesis through the ERα/ROCK-2 pathway.[17] Therefore, angiogenesis promoted by paeoniflorin may be involved in the inhibition of PTX-induced blood flow.

In conclusion, we demonstrated that PTX induced muscle weakness and atrophy in mice, and a traditional herbal medicine, SKT, inhibited these actions induced by PTX. In addition, PTX elicited a decrease in peripheral blood flow, which may have been due to muscle atrophy induced by PTX. PTX elicits a sensation similar to the numbness experienced in *seiza* (sitting in a kneeling position). This sensation may be involved in the decrease in the peripheral blood flow. Treatment with PTX elicits spontaneous dysesthesia, which is an abnormal or unpleasant sensation. Our previous report showed that PTX elicits spontaneous peripheral nerve firing, and SKT inhibits firing in mice.[11] Taken together, SKT may be useful for the treatment of PTX-induced peripheral neuropathy.

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Conflict of interest The authors declare no conflict of interest.

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