

Quercetin Attenuates Neuropathic Pain in Rats with Spared Nerve Injury

Noriko Muto, Yoshikazu Matsuoka*, Kyosuke Arakawa, Masako Kurita,
Hiroki Omiya, Arata Taniguchi, Ryuji Kaku, and Hiroshi Morimatsu

*Department of Anesthesiology and Resuscitology, Okayama University Graduate School of Medicine,
Dentistry, and Pharmaceutical Sciences, Okayama 700-8558, Japan*

Quercetin is a flavonoid widely found in plants and marketed to the public as a supplement. Several studies have reported its effect on glial cells. This study aimed to examine the effect of quercetin on the development of neuropathic pain and the underlying mechanism in a spared nerve injury (SNI) rat model. Male Sprague-Dawley rats randomly assigned to the control or the quercetin group were subjected to SNI of the sciatic nerve. We measured pain behaviors on the hind paw and glial fibrillary acidic protein (GFAP) in the dorsal root ganglion (DRG) and spinal cord. Oral administration of 1% quercetin, begun before surgery, attenuated mechanical allodynia compared to the control group at days 7 and 10 after SNI. On the other hand, established pain was not attenuated in a post-dose group in which quercetin was begun 7 days after SNI. Quercetin inhibited GFAP in the satellite glial cells of the ipsilateral L5 DRG on day 7 compared to the control group. Quercetin suppressed the development of neuropathic pain through a mechanism partly involving the inhibition of satellite glial cells. As its safety is well established, quercetin has great potential for clinical use in pain treatment.

Key words: flavonoid, dorsal root ganglion, glial fibrillary acidic protein, alternative medicine

Neuropathic pain is a chronic form of pain that is characterized by spontaneous onset, allodynia, and hyperalgesia. Chronic pain can persist for months at a time and can be very difficult to manage or treat effectively. Although various drugs, such as anti-depressants, anti-convulsants, and opioids, have been used for analgesic effect, they either have insufficient potency or produce adverse reactions. The development of novel drugs for neuropathic pain with fewer side effects and adequate efficacy has therefore been a focus of expectation. To achieve this goal, a great quantity of research has been conducted and various candidate compounds have been identified. Thousands of compounds have been shown to be effective in pre-clinical trials, but only a limited number have been tested in humans. The first obstacle to progressing from

cell and animal to human studies is safety. Even if a candidate compound could overcome this issue, patients would in the end have to bear the great cost burden of the time and resources required to prove the safety of the form synthesized for human trials. One strategy to avoid this obstacle is the adaptation of existing approved drugs or compounds, such as the above-mentioned anti-depressants and anti-convulsants, which were originally developed for other diseases. Known as “drug re-positioning,” this approach is now receiving attention.

Flavonoids are a group of plant metabolites consisting of up to 6,000 different compounds which are responsible for the vivid colors of plants [1]. Quercetin, a flavonoid widely found in fruits and vegetables (e.g., onions, cranberries, apples), is known to have anti-oxidant, anti-inflammatory, and anti-nociceptive effects,

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*Corresponding author. Phone: +81-86-235-7327; Fax: +81-86-235-6984
E-mail: matsuoka2@okayama-u.ac.jp (Y. Matsuoka)

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and is marketed to the public as a supplement for the alternative treatment of allergies, asthma, arthritis, gout, hypertension, and neurodegenerative disorders. However, there is a lack of data to provide a scientific basis for the relevant treatment claims. Epidemiological studies have demonstrated significant inverse correlation between intake of flavonoids and cardiovascular disease. Recently, several studies have reported that quercetin reduces blood pressure in hypertensive animals and humans [2]. Quercetin has also been found to suppress inflammatory pain [3] and diabetic neuropathic pain [4]. However, the mechanisms involved in these effects have not been fully elucidated.

For many decades, most research on neuropathic pain focused on neuronal mechanisms. However, recent studies have demonstrated that glial cells also act as powerful modulators of pain [5] and indicate that they may be an attractive target for the treatment of chronic pain. Accordingly, several drugs that inhibit glial cells have been examined for their analgesic effect [6]. Quercetin is also reported to have effects on astrocytes [7], but the detailed mechanism is not clear. The aim of the present study was thus to examine the effect of quercetin on the development of neuropathic pain and to clarify the underlying mechanism.

Materials and Methods

Animals and drug treatment. All animal procedures were carried out in accordance with the Ethical Guidelines for the Investigation of Experimental Pain in Conscious Animals issued by the International Association for the Study of Pain [8]. The Board of Animal Care and Use Committee of Okayama University Medical School approved the study on July 30, 2014 (OKU-2014410).

In total, 61 male Sprague-Dawley rats (CLEA Japan Inc., Tokyo, Japan) weighing 150-240 g at the time of surgery were used in the study. The animals were housed in cages under a 12-12 h light-dark cycle with free access to food and water. A standard rodent diet (MF diet; Oriental Yeast Co., Tokyo, Japan) with or without quercetin (Carbosynth, Berkshire, UK) was prepared by the manufacturer and daily diet consumption was recorded. Rats were randomly assigned to the different experimental groups. The experimental rats were allowed to acclimatize to the facility for 5 days prior to surgery.

Surgical procedures. All the rats were placed under anesthesia with pentobarbital sodium (40 mg/kg intraperitoneally). Additional inhalation anesthesia with isoflurane was given as needed. Spared nerve injury (SNI) of the sciatic nerve [9] was performed as described previously with modification. Briefly, the left tibial nerve was exposed at mid-thigh level, ligated with 6-0 silk thread, and cut, while the common peroneal and sural nerves remained intact. After appropriate hemostasis was confirmed, the wound was closed. All procedures were performed under aseptic conditions.

Behavioral assessment. Pain behavior was assessed by the von Frey test before and at 1, 3, 7, 10, 14, and 21 days after surgery. Mechanical allodynia was measured via the hind paw withdrawal threshold (PWT) with von Frey filaments (Touch-Test Sensory Evaluator; North Coast Medical, Morgan Hill, CA, USA). The rats were placed individually in a plastic cage (13×10×15 cm³) with an elevated wire mesh bottom (openings 5×5 mm²), allowing full access to the plantar surfaces of both hind paws. Mechanical stimuli were applied to the lateral plantar aspect of each hind paw with one of a series of nine von Frey filaments (0.4, 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, and 15.0 g). Each trial was started with a von Frey force of 2 g for 1-2 sec. The stimuli were given at intervals of at least several seconds, allowing for apparent resolution of any behavioral responses to previous stimuli. On the basis of the response pattern and the force of the final filament, the 50% PWT was determined by the up-down method of Dixon [10] and calculated using the formula described by Chaplan *et al.* [11]. If the strongest filament did not elicit a response, the PWT was recorded as 15.0 g.

Cell culture. The C6 rat glioma cell line was obtained from American Type Culture Collection (Manassas, VA, USA; catalogue no. CCL-107). The cells were thawed and maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin (Wako, Osaka, Japan), in an incubator containing 5% CO₂ at 37°C. Quercetin dissolved in dimethyl sulfoxide (DMSO) was added at final concentrations of 0 (vehicle), 1, 10, 30, and 50 μM in equalized volumes. After 1 h, cells were stimulated with 1 ng/ml of recombinant rat interleukin-6 (IL-6; R&D Systems, Minneapolis, MN, USA) for 3 h and 10 h to measure phospho-signal transducer and activator of transcription 3 (STAT3) and

glial fibrillary acidic protein (GFAP), respectively.

Western blotting. For *in vivo* study, rats were anesthetized deeply with pentobarbital and perfused transcardially with 50 ml of saline 7 and 14 days after SNI. The bilateral L5 dorsal root ganglions (DRGs) and spinal cords were excised and processed for western blotting. For *in vitro* study, cells were harvested with cell lysis buffer after IL-6 stimulation for 3 and 10 h. Total protein concentrations were measured with a BCA protein assay kit (Pierce, Rockford, IL, USA). The proteins were denatured and reduced by boiling in SDS and β -mercaptoethanol-containing buffers. They were then electrophoresed and separated on gradient polyacrylamide gels (Any kD precast gel; Bio-Rad, Hercules, CA, USA) and transferred onto nitrocellulose membranes. The membranes were first labeled with anti-GFAP antibody (1 : 5000, #2389; Cell Signaling Technology, Beverly, MA, USA), anti-phospho-STAT3 antibody (1 : 2000, #9145; Cell Signaling Technology), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1 : 100000, #G8795; Sigma Aldrich, St. Louis, MO, USA) as a loading control for 2 h at room temperature. After washing, horseradish peroxidase-conjugated donkey anti-rabbit (1 : 5000, NA934; GE Healthcare UK, Buckinghamshire, UK) and donkey anti-mouse IgM (1 : 3000, #sc-2064; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies were added. The membranes were then imaged using the enhanced chemiluminescence procedure (Bio-Rad). Densitometry was performed using Image Lab (Bio-Rad), and protein levels were expressed as values relative to the controls.

Immunohistochemistry. The rats were anesthetized deeply with pentobarbital and perfused transcardially with 50 ml of saline followed by 500 ml of 4% formalin 14 days after SNI. The bilateral L5 DRGs were excised and post-fixed in the same fixative for 2 h and incubated in phosphate-buffered 30% sucrose overnight. These tissues were embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and 16 μ m-thick frozen sections were cut and incubated overnight at 4°C with rabbit anti-GFAP antibody (1 : 1000, #12389; Cell Signaling Technology), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1 : 200) and AlexaFluor 488-labeled tyramide (1 : 200) according to the manufacturer's instructions (Life Technologies, Eugene, OR, USA). Nuclei were visualized with DAPI (ProLong Gold

Antifade Mountant with DAPI; Life Technologies).

Images were captured with a fluorescent microscope (EVOS FL Cell Imaging System; Life Technologies, Carlsbad, CA, USA) equipped with 20 \times objective (numerical aperture = 0.45).

Statistical analysis. Behavioral changes and GFAP level were assessed as primary and secondary experimental outcomes, respectively. Data are expressed as the mean \pm standard error of the mean (SEM). For behavioral assessment data, statistical analysis was performed with two-way measurement analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. Differences were considered statistically significant at a *p*-value of <0.05. For western blotting analysis data, statistical significance was determined with one-way ANOVA followed by Bonferroni's post-hoc test. For food intake data, statistical analysis was performed with Student's *t* test. A *p*-value of <0.05 was considered significant.

Results

Behavioral assessments. First, we tested the prophylactic effect of quercetin on the development of neuropathic pain. In the quercetin pre-dose groups, rats were given MF diet containing 1% or 0.1% quercetin from 4 days before surgery until sacrifice. The average intake of the diet is shown in Table 1. The average intake of quercetin was 229 \pm 13.0 (standard deviation) mg/day in the 1% quercetin group. In the control group, rats were given normal MF diet, in which the quercetin content was under the limit of detection. At baseline, there was no significant difference in mechanical withdrawal thresholds across the groups (Fig. 1A, B). After SNI, all rats developed ipsilateral tactile allodynia (Fig. 1A), while the contralateral PWT did not vary significantly from baseline values (Fig. 1B). In the 1% quercetin pre-dose group, mechanical allodynia was attenuated compared to the control group with signifi-

Table 1 The daily intake of food and quercetin

	Control group (n = 5)	Quercetin group (n = 5)	<i>p</i> -value
Food intake (g/rat/day)	19.9 \pm 0.3	22.9 \pm 0.6	< 0.01
Quercetin intake (mg/kg/day)	–	808 \pm 21	

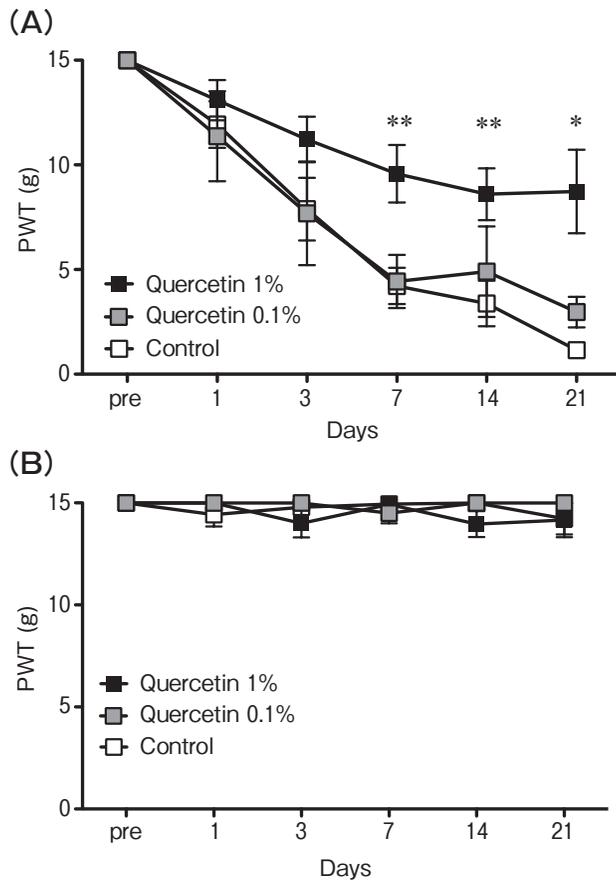


Fig. 1 Effect of quercetin on SNI-induced pain behavior. Time course of ipsilateral (A) and contralateral (B) 50% paw withdrawal threshold (PWT) in response to mechanical stimuli after SNI in the control group (white box, $n = 6-17$), 1% quercetin group (black box, $n = 9-20$) and 0.1% quercetin group (gray box, $n = 6$). Statistical analysis was performed with two-way measurement ANOVA followed by Bonferroni's post-hoc test. Data are expressed as the means \pm SEM. * $p < 0.05$, ** $p < 0.01$ versus the control group.

cant differences at days 7, 14, and 21 after SNI (9.57 ± 1.37 vs. 4.22 ± 0.86 , 8.60 ± 1.24 vs. 3.38 ± 1.09 and 8.73 ± 1.99 vs. 1.15 ± 0.37 , respectively). On the other hand, no effect was observed in the 0.1% quercetin pre-dose group.

Next, we tested the reversal effect of a 1% quercetin diet. In this experiment, MF diet containing 1% quercetin was started from 7 days after SNI (1% quercetin post-dose group). In contrast to the pre-dose group, mechanical allodynia was not attenuated in this group (Fig. 2). No motor deficiency was observed in any rat during the whole experimental period.

Effect of quercetin on GFAP. Many reports have

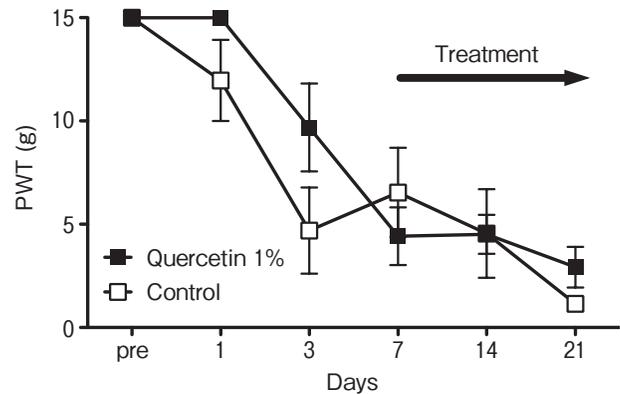


Fig. 2 Difference in quercetin effect on SNI-induced pain behavior (comparison of the timing of medication). Time course of ipsilateral 50% paw withdrawal threshold (PWT) in response to mechanical stimuli after SNI in the control group (white box, $n = 6$) and 1% quercetin post-dose group (black box, $n = 9$). Statistical analysis was performed with two-way measurement ANOVA. Data are expressed as the means \pm SEM. * $p < 0.05$ versus the control group.

demonstrated the anti-inflammatory effects of quercetin. In the present study, to identify the effect of quercetin on glial cells *in vitro* and to account for the correlation between quercetin administration and suppression of pain behavior, we measured the GFAP level in a C6 rat glioma cell line as a cellular astrocyte model. Although C6 cells constitutively expressed GFAP as well as primary cultured astrocytes, the level was so faint that we employed IL-6 to enhance GFAP expression, since IL-6 is a key activator of GFAP in astrocytes via the STAT3 pathway [12] and has been reported to play important roles in various pathological pain states. An inhibitory effect of quercetin on STAT3 activation was also reported previously [13].

The western blot analysis demonstrated that IL-6 increased the levels of GFAP and phospho-STAT3 (Fig. 3A). These changes were suppressed by quercetin dose-dependently. GFAP induction was decreased with 30 μ M of quercetin and was completely inhibited at 50 μ M of quercetin (Fig. 3A, B).

GFAP localization in DRGs. Satellite glial cells (SGCs) are glial cells in the peripheral nervous system that surround the individual neurons in the DRG. They are thought to play a role similar to astrocytes in the central nervous system and are suggested to be involved in the development of pathological pain [14, 15]. The use of GFAP as an astrocyte marker in the spinal dorsal horn has been well documented in many pain studies.

In the present experiments, immunohistochemistry was performed to localize GFAP in the DRG. GFAP was expressed predominantly in the SGCs of the ipsilateral L5 DRG in the rats with SNI (Fig. 4). No immunoreactivity was seen in the neuronal somata. GFAP immunoreactivities in the contralateral DRGs of both groups and in the ipsilateral DRGs of quercetin-treated rats were so faint that we performed western blot analysis to

quantitate them.

Suppression of GFAP in DRGs and spinal cord by quercetin treatment. Previous reports have demonstrated morphological changes in GFAP-immunoreactive cells and upregulation of GFAP content in the DRG after peripheral nerve injury [15, 16]. We tested whether quercetin treatment suppressed the induction of GFAP caused by SNI. Using western blot analysis,

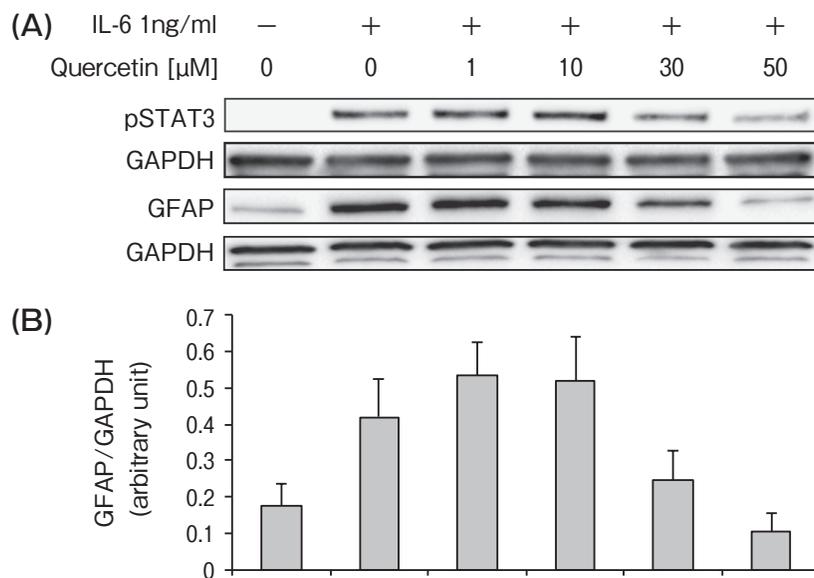


Fig. 3 Inhibition of GFAP synthesis by quercetin in C6 glioma cells. (A) Western blotting of whole cell lysates. Cells were treated with quercetin at the indicated concentrations 1h before interleukin-6 (IL-6) stimulation and harvested 3h (pSTAT3) and 10h (GFAP) after stimulation. (B) Densitometry of GFAP. Values are expressed as the GFAP densities relative to GAPDH as a loading control (n = 3). Data are expressed as the means \pm SEM.

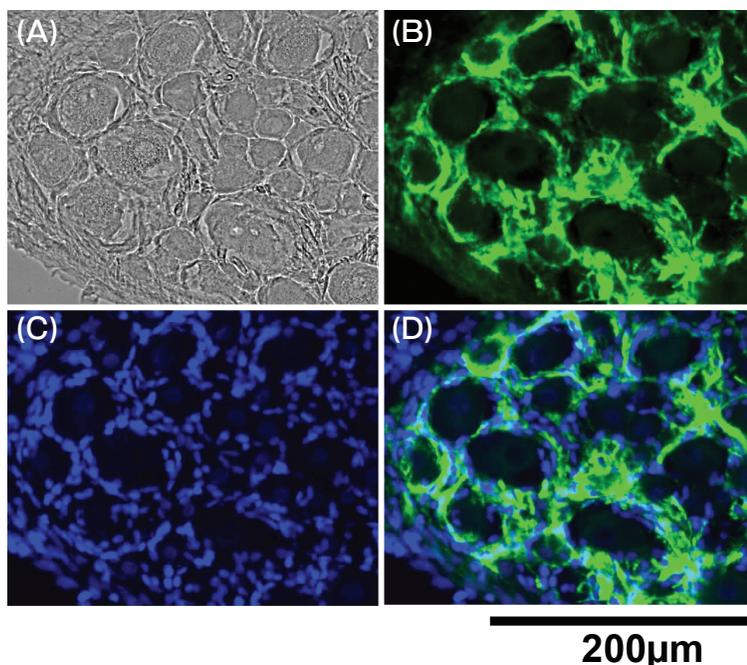


Fig. 4 Photomicrographs of the immunohistochemical localization of GFAP in DRGs. (A) Bright-field photograph of DRGs. Green (B) and blue (C) immunoreactivities show GFAP and nuclei, respectively. Nuclei surrounding neuronal cell bodies are those of SGCs. (D) Merged image of DRGs.

we measured GFAP content in the L5 DRGs and spinal cords of the SNI model with and without quercetin treatment. In the control group, GFAP was significantly induced in the ipsilateral L5 DRGs compared to the contralateral tissues on day 7 (Fig. 5A), with diminished induction on day 14 (Fig. 5B), as in a previous report [16]. In the quercetin group, rats were given MF diet containing 1% quercetin from 4 days before the

surgery until sacrifice. In the L5 DRGs, GFAP expression on the ipsilateral side was significantly inhibited by quercetin treatment on day 7 compared to the control group. GFAP expression on the contralateral side did not vary between the control and quercetin groups. We also performed western blotting on the L5 spinal cords. In both groups, there were no differences between the ipsilateral and contralateral tissues on days 7 (Fig. 6A)

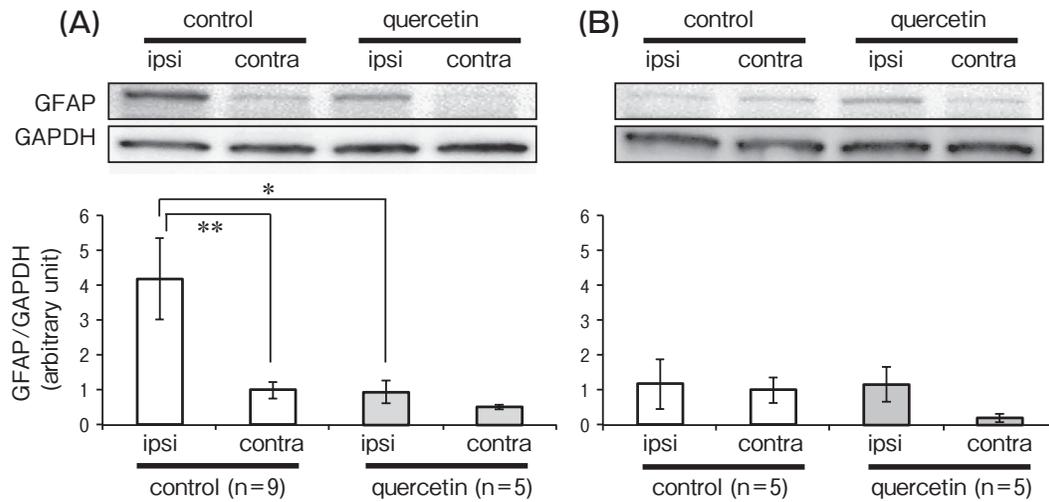


Fig. 5 Western blot analysis of GFAP protein expression in L5 DRGs with and without quercetin treatment. (A) Western blotting and statistical analysis of densitometry 7 days after SNI. * $p < 0.05$, ** $p < 0.01$. (B) Western blotting and statistical analysis of densitometry 14 days after SNI. GFAP protein expression was normalized by GAPDH as a loading control. Statistical analysis was performed with one-way ANOVA and Bonferroni's post-hoc test. Data are expressed as the means \pm SEM.

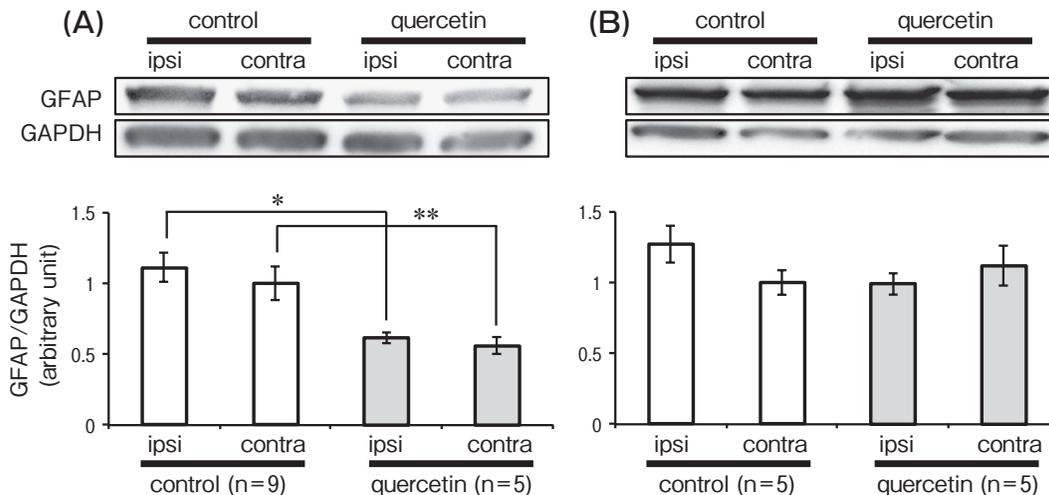


Fig. 6 Western blot analysis of GFAP protein expression in the L5 spinal cord with and without quercetin treatment. (A) Western blot bands and statistical analysis of densitometry 7 days after SNI. * $p < 0.05$, ** $p < 0.01$. (B) Western blot bands and statistical analysis of densitometry 14 days after SNI. GFAP protein expression was normalized by GAPDH as a loading control. Statistical analysis was performed with one-way ANOVA and Bonferroni's post-hoc test. Data are expressed as the means \pm SEM.

and 14 (Fig. 6B). GFAP expression was significantly inhibited on both sides by quercetin treatment compared to the control group on day 7. This tendency was not observed at 14 days after SNI in the L5 spinal cords.

Discussion

Quercetin has been reported to have a number of clinical actions [2,17-20], and its effects on pain have also been tested in different animal models [3,4,21]. A series of reports has focused on its effect on neuropathic pain, with a number of possible mechanisms suggested [22,23]. In the present study, we demonstrated the analgesic effect of quercetin using a rat SNI model. Our results showed that oral administration of quercetin prevented the development of the mechanical allodynia caused by nerve injury, whereas established neuropathic pain was not reversed. The preventive effect was correlated with GFAP inhibition in the L5 DRGs. These results indicate the importance of SGCs in the development of neuropathic pain.

Quercetin has been reported to have a number of favorable properties, including antioxidant, antinociceptive, and anti-inflammatory effects. The analgesic action of quercetin has been demonstrated in several previous studies. Quercetin inhibited nociceptive responses in capsaicin-, glutamate-, and formalin-induced pain models in mice [21]. The authors suggested that these effects occurred via the modulation of GABA_A, GABA_B, and 5-HT receptors, as well as the endogenous release of glucocorticoids. Anjaneyulu *et al.* reported that quercetin attenuates diabetic neuropathic pain in mice through modulation of the opioid mechanism [4].

One of the effects of quercetin is inhibition of GFAP expression in injured astrocytes [7]. GFAP is an intermediate filament protein that is expressed especially in astrocytes and is used as a marker of reactive glial cells [24]. GFAP is also found in SGCs, which are prominent glial cells in the DRG [25], and GFAP upregulation in SGCs after nerve injury has been reported [26-28]. Finally, it has been reported that spinal astrocytes are activated and play roles in various pain models [29]. In our present experiments, we observed increased GFAP levels in the bilateral spinal cords on day 7; however, because there was no laterality, which was observed in the behavioral assessments, we focused on SGC as a target of quercetin. The spinal GFAP activa-

tion was not seen on day 14. We considered that this finding was due to a return of the GFAP level to baseline, although in previous studies the time-course of spinal GFAP has varied depending on the pain models, ages, and species employed [30-32].

In addition to glial cells in the central nervous system, which are well known to be implicated in the induction and maintenance of neuropathic pain [29,33] SGCs in the sensory ganglia also contribute to neuropathic pain [15]. Following peripheral nerve injury, SGCs undergo changes in cell number, structure, and function [34]. Axotomy increases the gap junction and intercellular coupling of SGCs and decreases their membrane resistance [34], which results in neuropathic pain. SGCs also upregulate the production of proinflammatory cytokines such as tumor necrosis factor- α after peripheral nerve injury [35]. Our results suggest that quercetin's inhibition of SGCs, as demonstrated by GFAP suppression, attenuates pain behavior. However, further studies will be needed to elucidate the analgesic mechanisms of quercetin subsequent to GFAP suppression.

Quercetin is widely retailed and used as a supplement with a wide margin of safety. In a rodent trial, quercetin supplementation at a dose of 3-3,000 mg/day for 28 days resulted in no significant variations in standard toxicological parameters and unremarkable findings in the histopathological examination of various organs [36]. The bioavailability of quercetin following oral administration greatly influences its potential toxicity and beneficial effects. Our preliminary study showed a plasma quercetin concentration of 14.2 ± 5.1 μM in rats following 1% quercetin oral administration for 14 days. In humans, quercetin supplementation at doses of 500 mg/day and 1,000 mg/day over 12 weeks resulted in plasma quercetin concentrations of 1.38 ± 0.07 μM and 2.0 ± 0.1 μM , respectively [37]. Although there is a large divergence between our data and the reported plasma concentration in humans, quercetin intake may be affected by daily diet and supplementation [38]. Some of the therapeutic effects of quercetin, such as decreasing systolic blood pressure and reducing markers of oxidative stress and inflammation, have been reported at lower doses [17,39]. In addition, in clinical practice, the daily diet may affect the outcome of nerve injury. Shir *et al.* reported that dietary soy phytoestrogen included in rat chow suppressed neuropathic pain [40]. In this study we used the

MF diet as a basal chow. Although quercetin glycoside has been detected in the MF diet, the amount is vanishingly small [41]. We chose oral administration instead of intraperitoneal or intravenous administration not only due to the poor solubility of quercetin in solvents, but also because a previous report has shown that oral digestion with food increases quercetin bioavailability [42]. Accordingly, a number of approaches to increase the uptake and bioavailability of quercetin have been examined [38].

No analgesic effect of quercetin was observed in the post-dose group. Given this behavioral observation and the results of our western blotting, SGC activation seems to contribute to the initiation of pain processing rather than its maintenance. Many examples from the literature describing glial roles in chronic pain development suggest that microglia have an initiating role and astrocytes a maintaining role [43-44]. However, previous reports have mainly focused on these cells in the spinal dorsal horn. Evidence of early activation of SGCs after nerve injury has been reported. GFAP has been found to be upregulated within 24 h after axotomy [45] and SNI-induced GFAP upregulation to peak on days 3 to 6, then decline up to day 10 [16]. In the SNI model, pain behavior continues for more than 3 weeks [9]. Although inflammatory components are involved in the early postoperative period, it is generally accepted that neuropathic components are the major cause of continuous pain after 1 week. Because, in the present study, 1% quercetin attenuated pain behavior for 21 days compared with the control group, we can conclude that quercetin was effective against neuropathic pain. Taken together, our results highlight the importance of early SGC activation in the development of chronic pain states. Further experiments are needed to investigate the detailed mechanism of the signaling, including the role of cytokines and the gap junctions between SGCs and the neurons affected by quercetin administration.

In conclusion, prophylactic quercetin suppressed the development of neuropathic pain behavior through a mechanism partly involving the inhibition of SGCs. As its safety has been well established, quercetin has great potential for clinical use in pain treatment.

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