Acta Pharmacologica Sinica (2016) 37: 1166–1177
 1671-4083/16
www.nature.com/aps

Original Article

Quercetin ameliorates paclitaxel-induced neuropathic pain by stabilizing mast cells, and subsequently blocking PKCε-dependent activation of TRPV1

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Aim: Severe painful sensory neuropathy often occurs during paclitaxel chemotherapy. Since paclitaxel can activate mast cell and basophils, whereas quercetin, a polyphenolic flavonoid contained in various plants, which can specifically inhibit histamine release as a mast cell stabilizer. In this study we explore whether quercetin could ameliorate paclitaxel-induced neuropathic pain and elucidated the underlying mechanisms.

Methods: Quercetin inhibition on histamine release was validated *in vitro* by detecting histamine release from rat basophilic leukemia (RBL-2H3) cells stimulated with paclitaxel (10 μmol/L). In the *in vivo* experiments, rats and mice received quercetin (20, 40 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$) for 40 and 12 d, respectively. Meanwhile, the animals were injected with paclitaxel (2 mg/kg, ip) four times on d 1, 3, 5 and 7. Heat hyperalgesia and mechanical allodynia were evaluated at the different time points. The animals were euthanized and spinal cords and dorsal root ganglions were harvested for analyzing PKCε and TRPV1 expression levels. The plasma histamine levels were assessed in rats on d 31.

Results: Pretreatment with quercetin (3, 10, 30 μmol/L) dose-dependently inhibited excessive histamine release from paclitaxel-stimulated RBL-2H3 cells *in vitro*, and quercetin administration significantly suppressed the high plasma histamine levels in paclitaxel-treated rats. Quercetin administration dose-dependently raised the thresholds for heat hyperalgesia and mechanical allodynia in paclitaxel-treated rats and mice. Furthermore, quercetin administration dose-dependently suppressed the increased expression levels of PKCε and TRPV1 in the spinal cords and DRGs of paclitaxel-treated rats and mice. Moreover, quercetin administration may inhibited the translocation of PKCε from the cytoplasm to the membrane in the spinal cord and DRG of paclitaxel-treated rats.

Conclusion: Our results reveal the underlying mechanisms of paclitaxel-induced peripheral neuropathy and demonstrate the therapeutic potential of quercetin for treating this side effect.

Keywords: quercetin; paclitaxel; RBL-2H3 cells; heat hyperalgesia; mechanical allodynia; spinal cord; dorsal root ganglion; histamine; PKCε; TRPV1

Acta Pharmacologica Sinica (2016) 37: 1166–1177; doi: 10.1038/aps.2016.58; published online 8 Aug 2016

Introduction

Paclitaxel (brand name Taxol®) is an effective antineoplastic agent that has been used in breast, ovarian, lung, head and neck cancers since the 1990s[1] . Severe dose-limiting painful sensory neuropathy has been found to be associated with paclitaxel chemotherapy and frequently leads to the termination of treatment and contributes to the deterioration of the quality of life and a decrease in the survival rate of patients. Acute and chronic distal symmetrical paresthesias (occasionally with autonomic and motor dysfunctions) characterize paclitaxel-induced neuropathic pain[2], but the underlying etiologies and mechanisms are poorly elucidated. The concept that paclitaxel can activate mast cell and basophils is based on clinical observations of pruritus and allodynia in 30%–40% patients during the early use of paclitaxel[2, 3].

Quercetin (QUE) is one of the polyphenolic flavonoids that is distributed in various plants and possesses remarkable cytoprotection, anti-platelet, antioxidant, antithrombosis and anti-

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Received 2016-01-12 Accepted 2016-04-21

carcinogen biological activities[4, 5]. Moreover, quercetin specifically functions as a mast cell stabilizer that inhibits histamine release[6, 7]. Investigations have demonstrated that quercetin inhibits the degranulation of rat mast cells in connective tissue, mucosa[6] and bone marrow[7] and in human mast cells in the lungs and intestines[8].

It has been reported that the histamine released by mast cells plays a vital role in the development of both thermal hyperalgesia and mechanical allodynia in mice[9–11]. Mast cells adjacent to sensory nerves release histamine upon activation, which may initiate the release of substance P from the sensory nerve terminals[10]. Substance P is a neuropeptide that is closely related to pain transmission and can be potentiated by the sensitization of transient receptor potential cation channel subfamily V member 1 (TRPV1)[11], which is considered to be involved in activation of PKC, primarily the protein kinase C epsilon isoform (PKCε).

Dorsal root ganglion (DRG) neurons, primarily small and medium sized neurons, participate in spinal nociceptive transmission via the release of pain neuromediators that project to different laminae of the dorsal horn of the spinal cord. These neurons are thought to be responsible for the transmission of nociceptive stimulation[12, 13]. Histamine sensitizes TRPV1 to heat stimuli in cultured DRG neurons after binding its receptor H1R[14]. The function of TRPV1 is augmented by many endogenous mediators following direct PKCε activation[15]. Specifically, the phosphorylation and activation of PKCε intensifies the responses of sensory neurons to noxious heat[16]. Previous studies have demonstrated that PKCε activation in small- and medium-sized DRG neurons alters the TRPV1 function and consequently leads to hyperalgesia. Our current study addressed whether quercetin exerts an analgesic effect on paclitaxel-induced neuropathy by stabilizing mast cells.

Materials and methods

Cell viability assay

Cell viability was determined with a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium (MTT) assay (Sigma-Aldrich Co LLC, St Louis, MO, USA). Rat basophilic leukemia (RBL-2H3) cells (Chinese Academy of Sciences, Beijing, China), which are a type of mast cell line that is extensively used to test mast cell stabilizers, were suspended and seeded at a density of 5×10^4 in a 96-well plate. The cells adhered to the wall after 24 h and were then cultured in serum-free medium. Three groups were established and received different concentrations of quercetin (3, 10 and 30 μmol/L). Each group had 5 parallel wells and a duplication a non-treated control group. The cells were incubated with quercetin for 24 h. Next, the cells in each well treated with MTT and cultured in an incubator for 4 h. The supernatant was exchanged for 150 μL of fresh dimethylsulfoxide (DMSO) in each well. The plate was then placed on a rotator for 15 min. The absorbance value was read at 570 nm. The ratios of the mean absorbance values of the quercetin-treated cells to the values of the control group subtracted from 1 were taken as the final cell viabilities.

RBL-2H3 cell histamine release

Measurements of the histamine released from the RBL-2H3 cells were used for the determination of degranulation. The cells were suspended and seeded at a density of 5×10^4 in a 96-well plate and cultured overnight at 37 °C in 0.5% CO_2. The following five groups (five wells for each group) were arranged: a vehicle control group, a paclitaxel model group, and three quercetin-treated groups (3, 10 and 30 μmol/L). After starvation in serum-free medium, different concentrations of quercetin dissolved in PBS containing DMSO at a final concentration of 0.1% were added to the corresponding quercetin-treated groups. The same volume of vehicle was added to the other two groups. After incubation of the plate for 24 h, the paclitaxel diluted in MEM medium was added to all groups except the vehicle control group at a final concentration of 10 μmol/L, and the groups were then cultured for 30 min. The supernatants were collected for histamine release detection using a rat HIS ELISA kit (AMEKO, Shanghai, China) according to the instructions. The absorbance value was read at 450 nm.

Animals and drug treatment

Twenty-eight adult male Sprague-Dawley (180–220 g) rats and twenty-eight Institute of Cancer Research (ICR) mice (22–25 g) were housed with sawdust bedding under a 12 h light–dark cycle with free access to water and food in accordance with the Provisions and General Recommendation of Chinese Experimental Animals Administration Legislation. All procedures described were approved by the Animal Ethics Committee of School of Chinese Materia Medica, China Pharmaceutical University.

The rats were randomly and evenly allocated into the following four group: a paclitaxel-injected model group, two quercetin-treated groups including a low- (20 mg/kg) and a high-dosage (60 mg/kg) group, and a vehicle control group. With the exception of the vehicle control group, all groups were intraperitoneally (ip) injected with 2 mg/kg paclitaxel (Paclitaxel Injection®, Tai Ji, Chengdu, China) diluted in 0.9% saline on four days (d 1, 3, 5, and 7). The quercetin-treated rats were intragastrically administered quercetin (20 and 60 mg/kg) once per day (from d 1) half an hour before the injections over 40 d. The selections of the paclitaxel and quercetin dosages were based on our pilot experiment. The rats in the vehicle control group were intragastrically administered a solution of sodium carboxymethylcellulose (CMC-Na). The rat experiment was performed to examine the chronic toxicity induced by paclitaxel and the therapeutic effect of quercetin.

The grouping methods and drug treatments of the mice were nearly identical to those applied to the rats. The only difference was that the mice in the quercetin-treated and vehicle control groups received quercetin and CMC-Na, respectively, for only 12 d, *ie*, the duration of the mouse experiment was 12 d. The murine experiment was established to examine the acute toxicity induced by paclitaxel and the therapeutic effect of quercetin.

Behavioral assays

The tips of the tails of the rats and mice were immersed in water baths at temperatures of 52.0 °C and 49.5 °C, respectively. The time until tail withdrawal was recorded. The average values of the three trials were determined and used as the final data. During the Von Frey tests, the rats (mice) were placed in a transparent Plexiglas cage with a wire mesh floor bottom. After acclimatization for 15 min, the hind paw of the rat (mouse) was stimulated with a Von Frey hair (Chicago, USA, pressure ranges from 1.4 g to 15.0 g for the rats and 0.14 g to 2.0 g for the mice) using the up-and-down test method[17].

Biochemical estimations

Estimation of the plasma histamine level

Three rats in each group were randomly selected for the plasma histamine level test on the 31th day. Blood samples were taken from the ophthalmic vein plexus, placed in Eppendorf tubes and centrifuged. The plasma samples were collected for histamine testing using a rat HIS ELISA kit according to the instructions.

Western blot analysis

Four rats and four mice from each group were deeply anesthetized with 10% chloral hydrate and decapitated. Tissue samples of the L4–L6 sections of the spinal cords (*ie*, the lumbar spinal cords) were obtained from the rats and mice, and the DRGs were removed from the rats only. Samples containing 10 μg of protein from the four groups were loaded into adjacent lanes of a 10% polyacrylamide gel. The proteins were separated by electrophoresis and blotted onto a PVDF membrane using Bio-Rad Trans-Blot in transfer buffer for 30 min. The membranes were incubated in 5% non-fat powdered milk diluted in Tris-buffered saline-Tween (TBS-T). After dividing the membranes into 3 pieces according to the different molecular weights of the target proteins, they were incubated with the sheep anti-PKCε polyclonal antibody (1:1000, R&D Systems), rabbit anti-TRPV1 polyclonal antibody (1:1000, Bioworld Technology, MN, USA) and rabbit anti-GAPDH monoclonal antibody (1:1500) as a loading control at 4 °C overnight. After washing three times, the membranes were incubated with sheep anti-mouse IgG (1:10000, Bioworld Technology) for PKCε or rabbit anti-mouse IgG (1:15000; Bioworld Technology) for TRPV1 and GAPDH for 2 h at room temperature. Finally, the membranes were washed with TBS-T several times and exposed on films using a BeyoECL Plus detection system (Biosky Biotechnology Corporation, Nanjing, China). The gray values were determined with the Lab Works software.

Immunohistochemistry

Three rats from each group were deeply anesthetized with 10% chloral hydrate and cardially perfused with 4% paraformaldehyde dissolved in 0.1 mol/L phosphate buffer saline (PBS). The L4–L6 lengths of the spinal cord (lumbar spinal cord) and the DRGs were removed from the rats. The tissue samples were post-fixed in identical fixative at 4 °C overnight and then subjected to graded dehydration via sequential immersion in 10%, 20%, and 30% sucrose until they sank to the bottom. The tissues were frozen at -80 °C in acetone and then sliced at a thickness of 6 μm and placed on slides. The sections were incubated in PBS containing 10% bovine serum albumin (BSA) and then treated with 0.15% Triton X-100 for 15 min. After washing three times, the sections were incubated in sheep anti-mouse IgG (15 μg/mL; R&D System) for PKCε or rabbit anti-mouse IgG (1:100; Bioworld) for TRPV1 in phosphate-buffered saline (PBS) containing 10% BSA overnight at 4 °C. The sections were washed three times in PBS and then incubated with the secondary antibody, *ie*, sheep anti-mouse (for PKCε, 1:500; Bioworld Technology) or anti-rabbit (for TRPV1, 1:500; Bioworld Technology) in PBS containing 10% BSA for 2 h at RT. After flushing, the horseradish peroxidase (HRP) reaction was developed with a DAB chromogenic agent kit. The air-dried sections were sequentially dehydrated in alcohol (70%, 80%, 90% and 100%). The control tissue sections exhibited no specific staining because the primary antisera were omitted.

Statistical analysis

The data were analyzed as the mean±standard deviation (SD) and processed using GraphPad Prism 5.0 software. The data from the behavioral tests were processed using two-way repeated analyses of variance (ANOVAs). *P* values less than 0.05 were considered statistically significant.

Results

Cytotoxicity of quercetin to RBL-2H3 cells

We used MTT assays to determine the effects of quercetin on RBL-2H3 cell viability. Different concentrations of quercetin (3, 10 and 30 μmol/L) were co-cultured with RBL-2H3 cells. We found that quercetin did not affect the viability of the RBL-2H3 cells up to 30 μmol/L as indicated by the lack of significant differences between the groups (Figure 1).

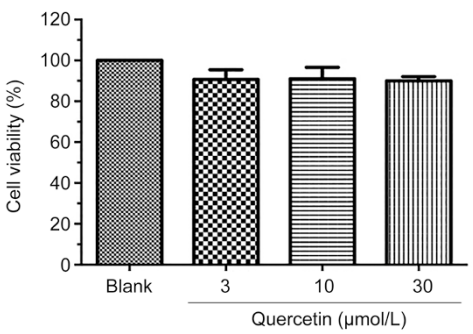

Figure 1. Cell viability was assayed by MTT. Viability of RBL-2H3 cells treated with quercetin in different concentrations (3, 10, and 30 μmol/L) showed no remarkable difference compared to blank. Data were analyzed as mean±SD. *n*=5.

Effects of quercetin on paclitaxel-induced histamine release by RBL-2H3 cells

As shown in Figure 2A, the levels of histamine release from

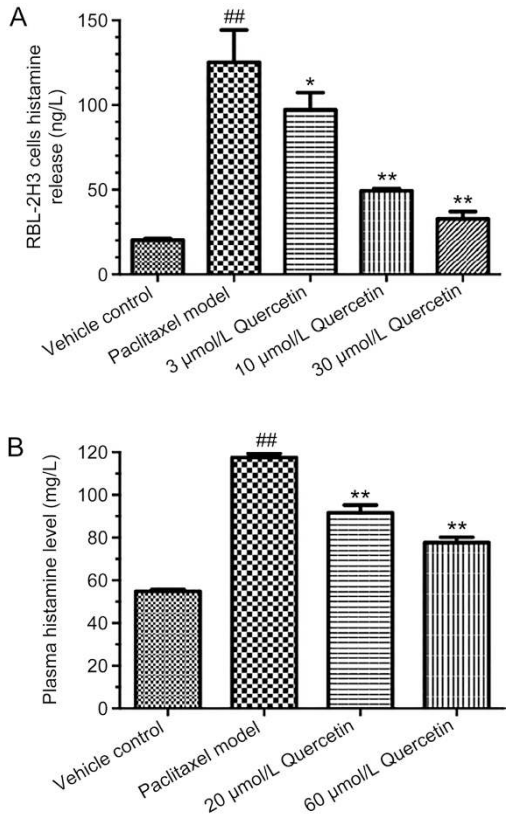

Figure 2. Histamine release of RBL-2H3 cells was assayed by HIS Elisa kit for rats (A). Histamine release of RBL-2H3 cells was significantly increased by paclitaxel which were dose-dependently inhibited by quercetin in concentration of 3, 10, and 30 μmol/L. Plasma histamine levels of rats were assayed by HIS ELISA kit for rats at d 31 (B). The error bars represent SD. Significant differences were observed in paclitaxel group versus vehicle control group. And the excess histamine level was remarkably decreased by quercetin. Data were analyzed as mean±SD; n=5 (A), n=3 (B). $^{*}P<0.05$, $^{**}P<0.01$ compared with paclitaxel model group. $^{\#}P<0.05$, $^{\#\#}P<0.05$ compared with vehicle control group.

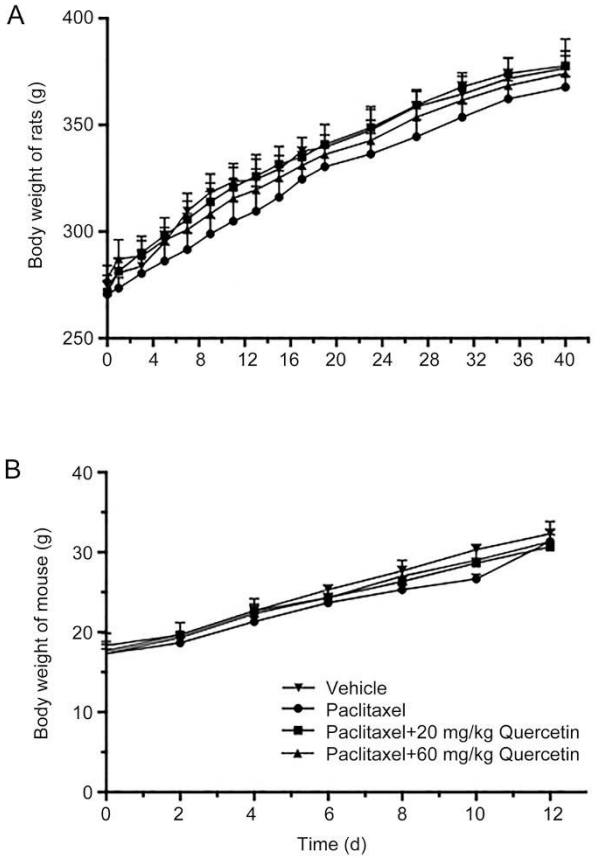

Figure 3. Mean body weights of rats (n=7) (A) and mouse (n=7) (B). Except for vehicle groups, animals from all groups got paclitaxel (2 mg/kg) on d 1, 3, 5, 7. Animals from quercetin groups were intragastric administrated with quercetin respectively in dose of 20 and 60 mg/kg every day from d 1. No significant difference was observed within groups.

the cells in the paclitaxel model group was significantly higher than that of the vehicle control group. However, the histamine release level was significantly and dose-dependently decreased in the cells in the quercetin-treated groups (3, 10 and 30 μmol/L) compared with the paclitaxel model group.

Estimation of the general toxicity

The rats and mice in the four groups were all alive and gained weight normally during the 40-d experiments. No significant differences of body weight was observed after four injections of Paclitaxel Injection® and during the treatment of quercetin as well (the vehicle control compared to other groups, P>0.05; Figure 3).

Effects of quercetin on the plasma histamine levels of the rats

The histamine level of the rats in the paclitaxel model group significantly differed from that of the vehicle control group. With the exception of the vehicle control group, the other groups were injected 2 mg/kg paclitaxel on d 1, 3, 5, and 7. The rats in the quercetin-treated group were dosed from d 1 to d 40. We noticed that the plasma histamine levels significantly decreased in a dose-dependent manner in the rats in the quercetin-treated groups compared with the paclitaxel model group (Figure 2B).

Effect of quercetin on heat hyperalgesia in the rats

At the baseline day (*ie*, d 0), there were no significant differences in the heat-stimulus withdrawal latencies between the groups of rats. Comparison of the paclitaxel injection group with the vehicle control group revealed significant decreases (P<0.05) in the tail withdrawal latencies from the second injection to the 40th day, which indicated the induction of peripheral neuropathy (Figure 4A). After the 20 mg/kg quercetin treatment, the rat tail withdrawal latencies were significantly increased beginning on the 8th day compared with the paclitaxel model group, and the rats that received the high dosage (60 mg/kg) exhibited significant differences from the 7th day (Figure 4A).

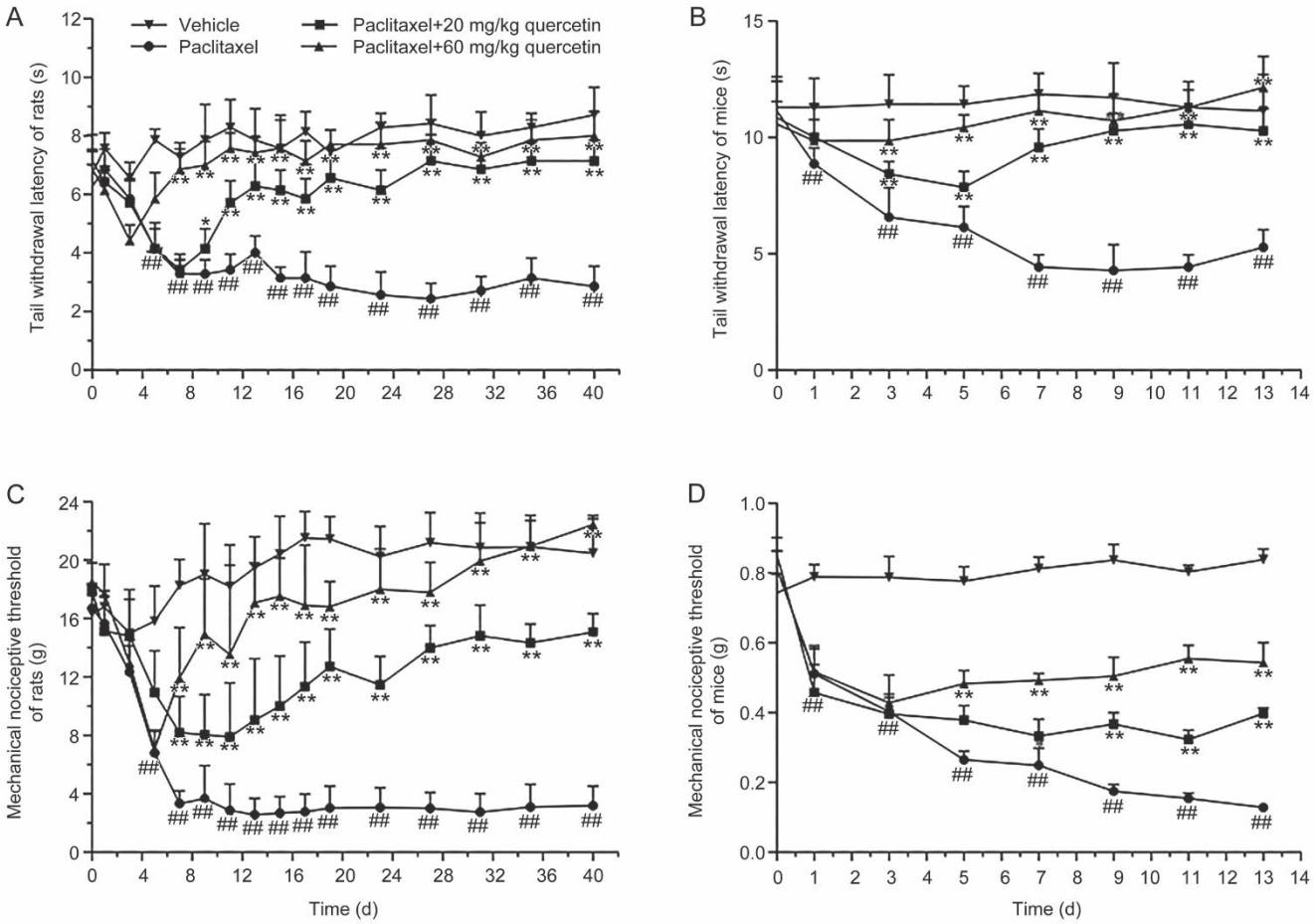

Figure 4. Effect of quercetin on paclitaxel-induced heat hyperalgesia of rats (A) and mice (B). Effect of quercetin on paclitaxel-induced mechanical allodynia of rats (C) and mice (D). Except vehicle control groups, other groups were injected 2 mg/kg paclitaxel on d 1, 3, 5, 7. Rats and mice of quercetin-treated groups were dosed (ip) with quercetin (20 and 60 mg/kg). Heat hyperalgesia and mechanical allodynia were tested at the different time points as indicated. Data were analyzed as mean±SD. n=7. $^{*}P$<0.05, $^{**}P$<0.01 compared with paclitaxel model group. $^{\#}P$<0.05, $^{\#\#}P$<0.05 compared with vehicle control group.

Effect of quercetin on heat hyperalgesia in the mice

On the baseline day, there were no significant differences in the heat-stimulus withdrawal latencies between the groups of mice. We found that the tail withdrawal latencies of the mice in paclitaxel model group were significantly decreased compared with the vehicle group (Figure 4B). After the 20 and 60 mg/kg quercetin treatments, the tail withdrawal latencies were significantly increased from the 3th day to the end of the experiment compared with the paclitaxel model group (P<0.05; Figure 4B).

Effect of quercetin on mechanical allodynia in the rats

No significant differences between the groups of rats in the mechanical nociceptive threshold were observed at baseline. The subsequent decreases in the mean paw mechanical nociceptive thresholds were significantly (P<0.05) and dose-dependently inhibited by the injections of quercetin (20 and 60 mg/kg) compared with the paclitaxel-injected model group from the 7th day, and this trend persisted until the completion of the test (Figure 4C).

Effects of quercetin on mechanical allodynia in the mice

No significant differences between the groups of mice in the mechanical nociceptive thresholds were observed at baseline. The paw mechanical nociceptive thresholds of the mice were significantly decreased beginning after the first injection of paclitaxel (P<0.05). After the 20 and 60 mg/kg quercetin treatments, the tail withdrawal latencies of the mice were significantly increased from the 7th and the 5th days, respectively, until the end of the experiment (P<0.05; Figure 4D).

Estimations of the PKCε levels in the spinal cords and DRG neurons of the rats

The anti-PKCε antibody labeled a protein band at approximately 87 kDa. In the paclitaxel-treated rats, the PKCε protein levels in the spinal cord (Figure 5A) and DRG (Figure 5C) neurons were significantly higher than those in the vehicle control groups (P<0.05). The expression levels of the PKCε protein

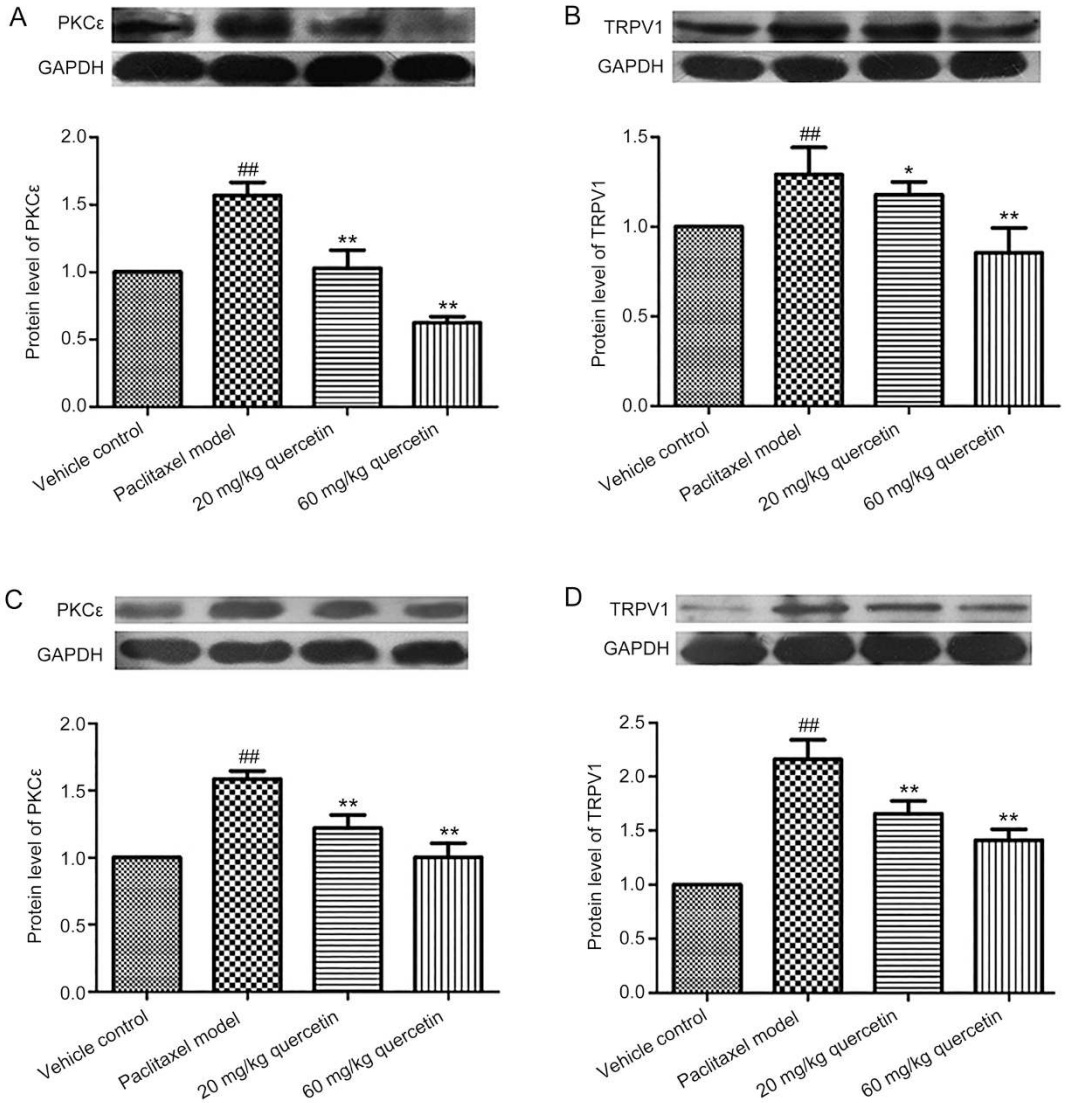

Figure 5. Western blot analysis of PKCε and TRPV1 in rat spinal cord (A) and (B) and DRG neurons (C) and (D). Grey values were processed as the percentage of the optical density (*OD*). Protein levels of PKCε and TRPV1 expressed in spinal cord and DRG neurons were presented as ratio of *OD* of the PKCε-positive bands or the TRPV1-positive bans to the GAPDH-positive ones. Data were analyzed as mean±SD. n=4. $^{*}P$<0.05, $^{**}P$<0.01 compared with paclitaxel model group. $^{\#}P$<0.05, $^{\#\#}P$<0.05 compared with vehicle control group.

in the spinal cords of the 60 mg/kg paclitaxel treatment group decreased by nearly two-thirds relative to the level in the paclitaxel model group (P<0.05), and the low dosage (20 mg/kg) group also exhibited a significantly lower PKCε expression level (P<0.05; Figure 5A). Quercetin moderately and dose-dependently decreased the levels of PKCε expression in the DRG neurons compared with the model group (P<0.05; Figure 5C).

Estimation of the PKCε levels in the spinal cords of the mice

Paclitaxel led to a significant increase in expression of PKCε in the spinal cord compared with the vehicle control mice (P<0.05). After the quercetin treatments, the PKCε levels were significantly (P<0.05) and dose-dependently decreased in the quercetin-treated mice (20 and 60 mg/kg) compared with the model animals (P<0.05; Figure 6A).

Estimation of the TRPV1 levels in the spinal cords and DRG neurons of the rats

The anti-TRPV1 antibody labeled a protein band at approximately 89 kDa. In the paclitaxel-treated rats, the TRPV1 protein level in the spinal cord was significantly higher than that in the vehicle control groups (P<0.05). Notably, paclitaxel increased the TRPV1 levels in the DRG neurons by approximately two-fold relative to the vehicle groups (P<0.05). Quercetin significantly and dose-dependently decreased the expressions of TRPV1 in the DRG neurons (P<0.05; Figure 5D) and spinal cords (Figure 5B) compared with model rats.

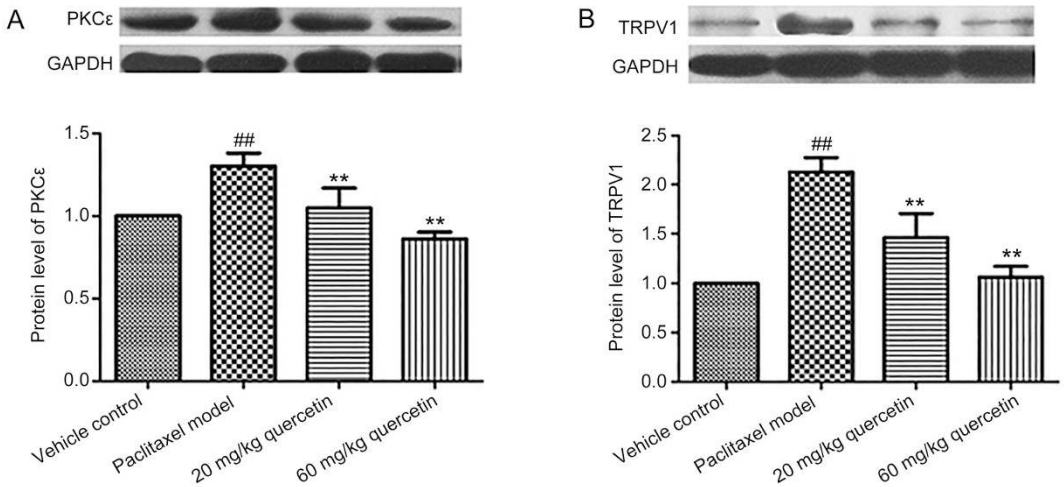

Figure 6. Western blot analysis of PKCε (A) and TRPV1 (B) in mice spinal cord. Grey values were processed as the percentage of the optical density (*OD*). Protein levels of PKCε and TRPV1 expressed in spinal cord were presented as ratio of *OD* of the PKCε-positive bands or the TRPV1-positive bans to the GAPDH-positive ones. Data were analyzed as mean±SD. n=4. $^{*}P$<0.05, $^{**}P$<0.01 compared with paclitaxel model group. $^{\#}P$<0.05, $^{\#\#}P$<0.05 compared with vehicle control group.

Estimation of the TRPV1 levels in the spinal cords of the mice

Paclitaxel contributed to a greater than two-fold increase in expression of TRPV1 in the spinal cord compared with the vehicle control group of mice (P<0.05). The TRPV1 level was significantly (P<0.05) and dose-dependently decreased in the mice in the quercetin-treated groups (20 and 60 mg/kg) compared with the paclitaxel group (P<0.05; Figure 6B).

Estimations of the PKCε and TRPV1 distributions in the spinal cords of the rats

Figure 7 illustrates that both PKCε and TRPV1 were mainly expressed in the spinal dorsal horns of the rats in each group. Remarkable increases in the PKCε- and TRPV1 immunoreactive profiles (mean grey values) were observed in the dorsal horns of the lumbar spinal cords of the paclitaxel model rats (Figure 7Ab and 7Bb) compared with the vehicle control group (Figure 7Aa, and 7Ba). Forty days of both the low- (Figure 7Ac and 7Bc) and high- (Figure 7Ad and 7Bd) dose quercetin treatments significantly and dose-dependently reduced the mean grey values for PKCε and TRPV1 in the spinal dorsal horns (P<0.05). The high dosage of quercetin also decreased the expressions of both PKCε and TRPV1 to the normal levels. PKCε translocated from the cytosol to the membrane in the spinal cord neurons of the paclitaxel model rats, which did not occur in the rats in the control or quercetin-treated groups (Figure 8).

Accordingly, it is believed that the terminals of the spinal nerves and primary afferent nerves contained PKCε and TRPV1.

Estimation of the PKCε and TRPV1 distributions in the DRG neurons of rats

To determine the locations of PKCε and TRPV1, lumbar DRGs were coated with antibodies. PKCε and TRPV1 immunoreactivities were observed in large areas of the DRG neurons of the paclitaxel model rats (Figure 9Ab and 9Bb) and remarkably exceeded the immunoreactive areas observed in the vehicle control group (Figure 9Aa and 9Ba). Forty days of quercetin treatment at both the low (Figure 9Ac and 9Bc) and high (Figure 9Ad and 9Bd) dosages significantly decreased the immunoreactive areas of both the PKCε and TRPV1 proteins (P<0.05).

We noted that PKCε was widely found to be translocated from the cytosol to the membrane in the DRG neurons from the paclitaxel model rats (Figure 9A, marked with black arrows), which was not observed in the vehicle control group and minimal observed in the quercetin-treated groups.

Discussion

Paclitaxel is a generally used chemotherapeutic medicine that has the ability to prevent the division of tumor cells by halting mitosis. Undesirable side effects, such as hyperalgesia and allodynia, often occur during chemotherapy due to the non-selective properties of this medicine. The dose-dependent neuropathic pain that paclitaxel elicits is characterized by numbness, tingling, burning sensations, and other symptoms that significantly limit the clinical use of paclitaxel[18]. To date, few effective drugs have been developed to solve this problem.

Several rodent models have been employed to investigate the symptoms and underlying mechanisms of paclitaxel-induced mechanical allodynia and thermal hyperalgesia[19–21] because paclitaxel-induced peripheral neuropathy in rats exhibits characteristics that are similar to those of human beings[22]. In the present animal model, paclitaxel induced rat hypersensitivity beginning on the 5th day after the first injection of paclitaxel, and interestingly, the mice exhibited neuropathic pain symptoms almost immediately after the injection.

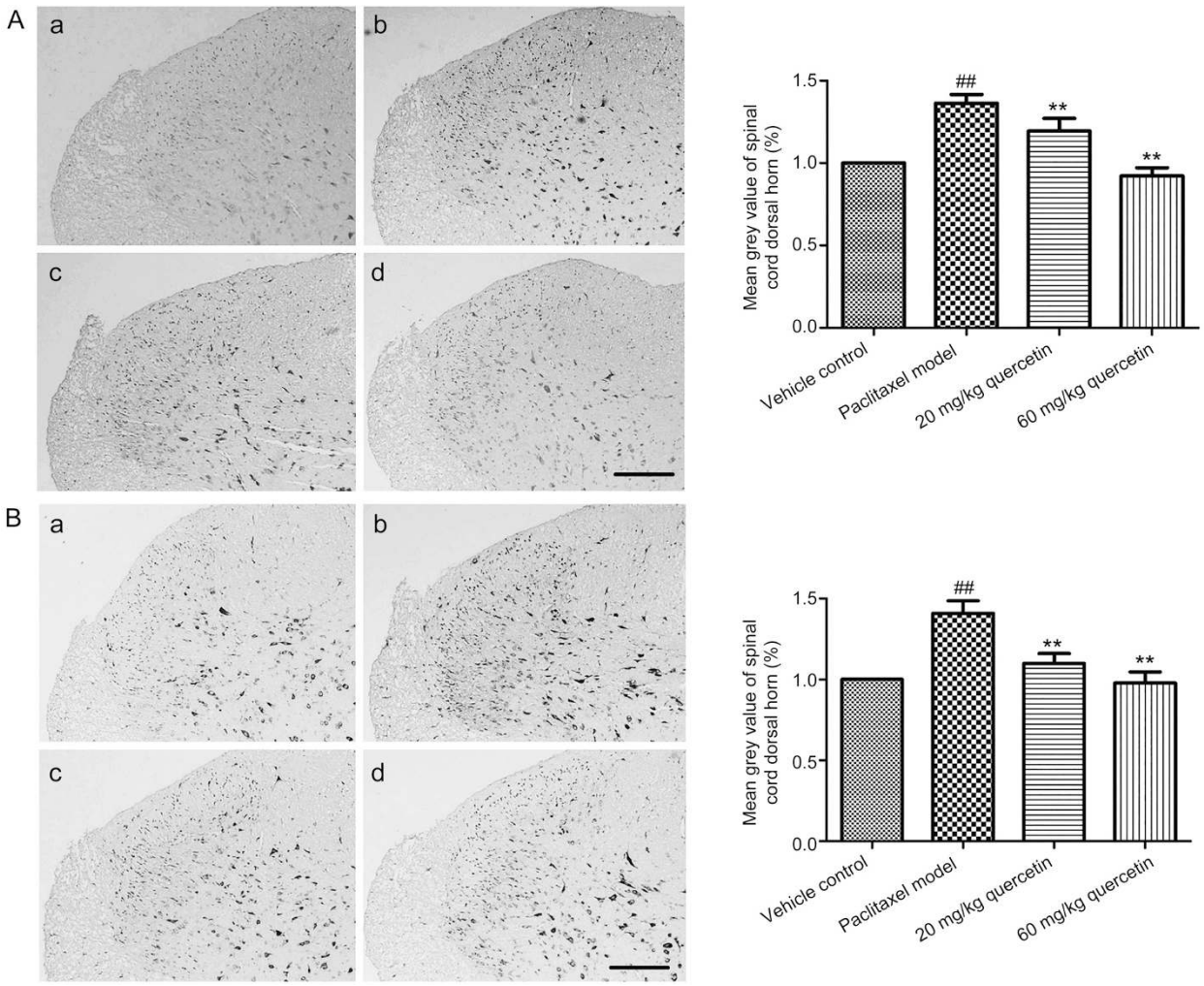

Figure 7. Immunohistochemistry assay of PKCε (A) and TRPV1 (B) in dorsal horn of rat's lumbar spinal cord from vehicle control (a), paclitaxel model (b), low (c) and high (d) dosage of quercetin. Mean grey value of spinal cord dorsal horn was analyzed as mean±SD. n=3. $^{*}P$<0.05, $^{**}P$<0.01 compared with paclitaxel model group. $^{\#}P$<0.05, $^{\#\#}P$<0.05 compared with vehicle control group. Scale bar=200 μm.

The rats and mice injected with paclitaxel were demonstrated to exhibit typical thermal hyperalgesia and mechanical allodynia. Previous evidence has demonstrated that the paclitaxel-induced rat peripheral neuropathy can persist for as long as 35 d[23]. A single dose of 16 or 32 mg/kg paclitaxel also causes a short-term nociceptive neuropathy in rats[24]. The present investigation revealed that long-term treatment with quercetin alleviated paclitaxel-induced chronic neuropathic pain in rats as illustrated by the finding that quercetin enhanced the thresholds for heat hyperalgesia and mechanical allodynia. Furthermore, the short-term delivery of quercetin mitigated the acute neuropathic pain produced by paclitaxel in mice.

The over-production of ROS and decreases in endogenous antioxidants are universally viewed as the major contributors to paclitaxel toxicity[25]. Recently, mast cell degranulation has been proposed to play a role in the neuropathic pain that results from paclitaxel therapy[26]. Mast cells are resident immune cells in the peripheral tissues and central nervous system (CNS). The former mast cells are adjacent to sensory nerve terminals and release histamine in a form of degranulation following inflammatory stimuli, which ultimately leads to the release of neuromodulators (*eg*, SP, CGRP, *etc*) from nociceptive fibers[27]. Chemical mediators released from mast cells through degranulation activate nociceptors that can conversely release mediators to activate mast cells when injured[28, 29]. When compared to wild-type (WT) mice, mast cell-deficient mice exhibit hypo-responsive hind paws in the presence of heat and mechanical stimulus[30]. The mast cell-dependent nociceptive response has been reported to be related to histamine signaling[31]. A large amount of evidence supports the notion that mast cells contribute to pain pathologies regardless of whether they occur in biomedical exploration or in clinical practice. Hyperalgesia is an excessive pain response that can be caused by the histamine released by mast cells[32]. The results of our experiments revealed that an increase in the

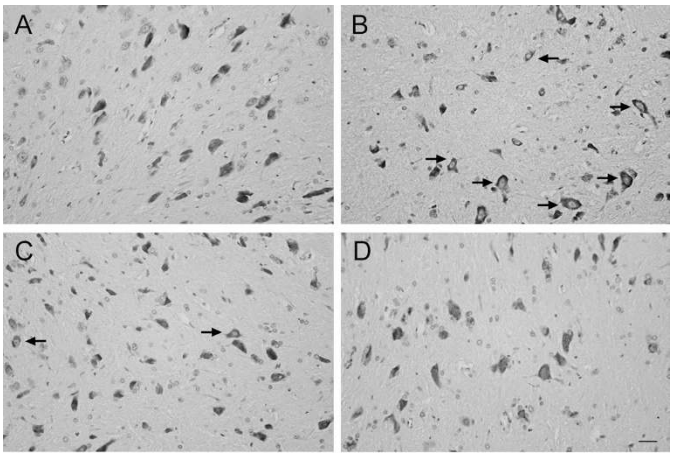

Figure 8. Immunohistochemistry assay of PKCε in lumbar spinal cord of rat from vehicle control (A), paclitaxel model (B), low (C) and high (D) dosage of quercetin groups. Black arrows point to PKCε translocation positive cells. Scale bar=20 μm.

plasma histamine level occurred in the rats together with heat hyperalgesia and mechanical allodynia, which supports the contribution of histamine to paclitaxel-evoked neuropathic pain.

Many previous findings have demonstrated that quercetin can inhibit degranulation in various types of mast cells, including rat peritoneal[33] and intestinal mast cells[6], RBL-2H3 cells[34], human mast cell lines[35, 36] and human basophils[37]. A previous *in vivo* study indicated that the antioxidant activity of quercetin prevents docetaxel-induced testicular damage in rats. Docetaxel and paclitaxel both belong to the taxane family[38]. However, there are no published reports that have investigated the mast cell-stabilizing capacities of quercetin in the treatment of paclitaxel-induced neuropathic pain. The *in vivo* and *in vitro* tests we performed both revealed the efficacy of quercetin in stabilizing the mast cell membrane and thereby inhibiting the excessive histamine release stimulated by paclitaxel. This first-time validation suggests the possibility that quercetin attenuates paclitaxel-induced neuropathic pain via action on mast cells.

To test the hypothesis further, we detected the downstream proteins that have been proven to be activated by histamine (a main mediator that exists in mast cell secretory granules). There are reports that have certified that increased histamine levels evoke Ca^{2+} influxes that are involved in TRPV1 expression in the DRG neurons of rats[39] and mice. Nevertheless, it has also been documented that a great multitude of cells in the rat DRG are sensitive to neither histamine nor capsaicin[40]. Among the histamine receptors, H1R is the one that strengthens the influence of histamine on TRPV1, and the sensitization of this receptor is dependent on the activation of PKC[41]. TRPV1 is acknowledged to exist in peripheral nerve terminals and can be sensitized by noxious heat over 43 °C. Additionally, some evidence also validates the contribution of TRPV1 to mechanotransmission after injuries in different conditions[42–44]. There are three isoforms of PKC on nociceptors that colabel with TRPV1[45]. Among these isoforms, PKCε is the most dominant in paclitaxel-induced neuropathy[18]. The activation of PKCε by other inflammatory mediators, such as bradykinin, has been proven to enhance the sensitivity of nociceptive neurons to heat stimulation[44]. Moreover, PKCε has been identified as the isoform of PKC that is likely closest to the onset of mechanical hyperalgesia[44], which has been further validated by Hucho[46]. Dutra *et al* demonstrated that a cumulative dose of 10 mg/kg paclitaxel causes mechanical hypersensitivity in mice, the pathogenesis of which is the up-regulation of PKCε in the spinal cord and the DRGs. Moreover, a PKCε inhibitor is capable of reducing this hypersensitivity[47].

It was clear from the Western blot assays that paclitaxel greatly increased the TRPV1 and PKCε expression levels in both the spinal cords and DRGs of the rats and mice. These results might explain the short withdrawal latencies of the animals in the model group during the tail immersion and Von Frey tests. The excessive expression of TRPV1 was strongly inhibited by quercetin, which indicates that quercetin might relieve paclitaxel-produced thermal hyperalgesia through the down-modulation of TRPV1 and PKCε. The PKC blocker hypericin has been proven to attenuate NO-induced nociceptive hypersensitivity, and this decrease is accompanied by a decreased expression of PKCε[48]. In a mouse model of paclitaxel-induced neuropathy, increased mast cell tryptase activity in the spinal cord, DRGs and peripheral tissues have been observed to be correlated with the repetitive administration of paclitaxel. Furthermore, sensitized TRPV1 and overactivated PKCε were also detected at the same site[49]. Therefore, the activation of the relevant proteins that are stimulated by mast cell degranulation may be correlated with paclitaxel-induced neuropathy. An *in vitro* test demonstrated that PKCε in DRG neurons translocates from cytoplasm to the membrane following the application of paclitaxel. Furthermore, calcitonin gene-related peptide (CGRP) is widely considered to be a critical pain transmitter and has been proven to be upregulated by paclitaxel. The inhibition of PKCε and subsequent restoration of the CGRP level explains its role in pain transmission[45]. However, the problem of whether the upregulation of PKCε contributes to neuropathic pain remains unsolved.

In the peripheral system, PKCε and TRPV1 have been extensively considered to participate in the modulation of the nociceptive process and to act on the nociceptive neurons that are distributed in the DRGs and spinal cord[50, 51]. Hence, it is reasonable that PKCε and TRPV1 may exist in these types of neurons. The current immunocytochemistry results verified that both of the proteins are expressed in the DRG neurons and the spinal cord dorsal horn.

The mean grey values from the immunohistochemical stainings revealed that paclitaxel increased the levels of PKCε and TRPV1 in the rat spinal cord dorsal horns and DRGs. These increases were persuasively inhibited by quercetin and nearly remained at normal levels at the high dosage. As can be observed from Figure 8, paclitaxel stimulated the translocation of PKCε from the cytoplasm to the membrane in the neurons

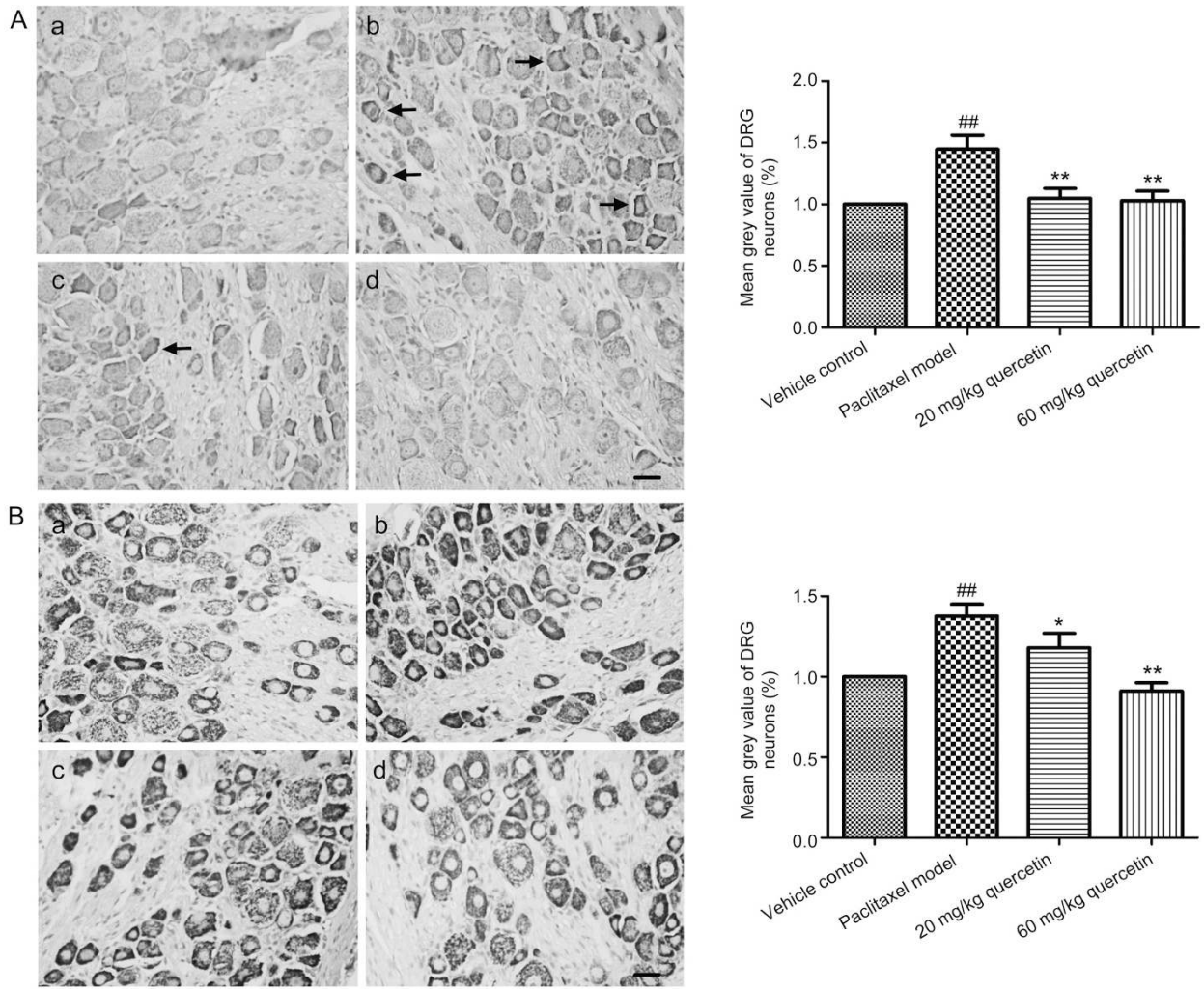

Figure 9. Immunohistochemistry assay of PKCε (A) and TRPV1 (B) in lumbar DRG neurons of rat from vehicle control (a), paclitaxel model (b), low (c) and high (d) dosage of quercetin groups. Black arrows point to PKCε translocation positive cells. Mean grey values of PKC ε-positive or TRPV1-positive cells were analyzed as mean±SD. n=3. $^{*}P$<0.05, $^{**}P$<0.01 compared with paclitaxel model group. $^{\#}P$<0.05, $^{\#\#}P$<0.01 compared with vehicle control group. Scale bar=20 μm.

of the rat spinal cord. This type of PKCε activation was strikingly suppressed by quercetin at both the low and high dosages.

In conclusion, quercetin attenuated both acute and chronic paclitaxel-induced neuropathic pain by stabilizing the mast cell membrane, which inhibited the excessive histamine release. Subsequently, the activation of the downstream proteins PKCε and TRPV1 was blocked by quercetin. These findings demonstrated the underlying mechanisms of paclitaxel-induced peripheral neuropathy and that quercetin could be an efficient drug for controlling this side effect.

Acknowledgements

This study was funded by the National Natural Science Foundation of China (Grant No 81072976 and 81173623) and the QingLan Project of Jiangsu Province (2014).

Author contribution

Fang HUANG and Zai-jie Jim WANG designed the experiments; Wei GAO performed the cell research and wrote the paper; Yan ZAN and Xiao-yu HU contributed to the animal experiments and analyzed the data.

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