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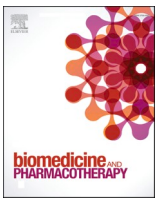

Vitexin inhibits pain and itch behavior via modulating TRPV4 activity in mice

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ABSTRACT

Itching and pain are distinct unpleasant sensations. The transient receptor potential cation channel subfamily V member 4 (TRPV4) pathway is regarded as a shared pathway that mediates pain and itching. Vitexin (Mujingsu, MJS), a C-glycosylflavonoid, is an effective analgesic. This study aimed to explore the antinociceptive and anti-pruritic effects of MJS and whether its effects are mediated via the TRPV4 pathway. Mice were treated with MJS (7.5 mg/kg) 0.5 h prior to the initiation of the pain or itch modeling process. The results showed that MJS suppressed pain-like behavior in hot plate, thermal infiltration, glacial acetic acid twisting, and formalin tests. Administration of MJS decreased the pruritus response induced by histamine, C48/80, chloroquine and BAM8-22 within 30 min. MJS reduced scratching bouts and lessened the wiping reaction of mice under TRPV4 activation by GSK101 (10 μg/5 μl). MJS inhibited scratching behavior in acetone–ether–water (AEW)-treated mice within 60 min. An H_1 receptor antagonist—chlorpheniramine (CLP, 400 mg/kg)—and a TRPV4 antagonist—HC067047 (250 ng/kg), exhibited similar effects to those of MJS. Moreover, MJS ameliorated dry skin itch-associated cutaneous barrier disruption in mice. MJS did not inhibit the expression of TRPV4 in the dorsal root ganglion neurons at L2–L3 in AEW mice. These results indicate that the analgesic and anti-pruritic effects of MJS in acute and chronic pain and itching, as well as itching caused by TRPV4 activation, could be attributed to the TRPV4 pathway modulation.

1. Introduction

Moderate acute pain and itching undoubtedly have a protective function in our body, but they can also negatively impact patients' physical, social, and psychological well-being, leading to deterioration in their quality of life. Long-term pain and itching can result in alterations to the related neural circuits, leading to pathological changes and nervous system disorders [1,2]. According to clinical case data, at least 20 % of patients with chronic pain lack effective treatment [3]. The safe and effective treatment of pain and itching has always been an urgent challenge in clinical settings. Accumulating evidence indicates that transient receptor potential (TRP) channels are involved in the transmission of various somatosensory stimuli, including itching, pain, temperature, and mechanosensation. TRPs are non-selective cation channels, and three subtypes, TRPV1, TRPV4, and TRPA1, play important roles in pain and different forms of pruritus [4–6].

TRPV4 is a TRP receptor that is activated within the range of 27–35 °C and can be activated by mechanical stimulation, low osmotic pressure, and GSK101, either subcutaneously or in the bloodstream [7]. TRPV4 knockout mice are less sensitive to thermal and mechanical pain stimulation, and TRPV4 blockers can relieve skin pain caused by sun exposure [7,8]. Pierre et al. identified a potential role of TRPV4 in the induction or persistence of sensory disturbances in paresthesia, cold dysesthesia, and painful disorders induced by neurotoxic shellfish poisoning syndromes [9]. Moreover, neuropathic sensation detection revealed a higher epidermal expression of TRPV4 in patients with chronic pruritus [10].

The study mainly used TRPV4 conditional knockout mice in sensory

Abbreviations: TRPV4, transient receptor potential cation channel subfamily V member 4; MJS, Mujingsu; AEW, acetone/ethanol/water; CLP, chlorpheniramine; DGR, dorsal root ganglion; i.v, intravenous injection; HE, hematoxylin & eosin; CQ, chloroquine.

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neurons and found that TRPV4 plays a significant role in histamine-induced itching [1]. The TRPV4 pathway is involved in the itching reaction caused by all histamine-dependent itching substances, including C48/80 and ET-1 [1,11]. Approximately 90 % of 5-HT-sensitive dorsal root ganglion (DRG) neurons were found to be immunoreactive to an antibody against TRPV4 [12]. Additionally, the TRPV4 pathway is involved in chloroquine (CQ)-induced histamine-independent pruritus [11]. Studies identified TRPV1 pathway as an auxiliary pathway of the TRPV4 pathway in the transmission of both histamine- and CQ-induced pruritus signals [6,11]. The acetone–ether–water (AEW) model is frequently used chronic itch model, in which the induced pruritus response has been shown to be associated with the TRPV1, TRPV4, and TRPA1 pathways [1,13].

There is a lack of effective drugs for treating pain and itching in clinical practice; morphine analgesics have a low safety profile and can be addictive, whereas H_1 receptor blockers and steroids are the most commonly used drugs in the treatment of itching, but have limited efficacy in many types of histamine-independent pruritus and are associated with a high rate of relapse [14,15]. Therefore, there is an urgent need to identify safe and broad-spectrum drugs to treat pain and itching. Vitexin (Mujingsu, MJS) is a C-glycosyl flavonoid with several pharmacological activities, including anti-nociceptive, anti-diabetic, anti-inflammatory, anti-myocardial ischemic and anti-viral effects [16–19]. MJS can inhibit the noxious reaction induced by capsaicin, a TRPV1 agonist [18]. Additionally, it displays behavior-specific antigenicity against postoperative pain mediated by opioid and GABAA receptors [19]. In our preliminary experiments, we discovered that MJS has an inhibitory effect on histamine and other pruritus models. In this study, we aimed to further explore the regulatory effect of MJS on experimental pain and itching, as well as its possible mechanism. Considering the important function of the TRPV4 pathway in the signal transmission of pain and itching, we focused on the role of MJS in the TRPV4 pathway using a chronic dry skin itch model.

2. Materials and methods

2.1. Animals

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Shenzhen Polytechnic, which comply with the National Institutes of Health Guidelines for the Care and Use of laboratory Animals in ARRIVE guidelines. C57BL/6J mice (6–8-week-old mice at 20–25 g body weight, SPF) were purchased from Jinan Pengyue Experimental Animal Breeding Limited. Mice were housed in animal laboratory (5 mice per cage, 21–23 °C, 40–70 % relative humidity, 12:12 h light/dark) with free available to water and food. All animals were fed adaptively for at least 48 h before the experiment.

2.2. Drugs

MJS was provided by Zhejiang Qixing Pharmaceutical Technology Co., Ltd. with batch no. 20201216 and purity of 99.5 %. MJS were dissolved in physiological saline in a concentration of 0.75 mg/ml. Mice were treated with MJS (7.5 mg/kg, i.v) or vehicle respectively 0.5 h before every acute nociception test or before all kinds of molding pruritogens injection except for the AEW treated mice.

2.3. Acute nociception tests

2.3.1. Hot plate test

Mice were placed on a hot plate maintained at 48 °C (± 0.5 °C). Two control latencies at least 10 min apart were determined for each mouse. The normal latency (reaction time) was approximately 15 s. The latency was also evaluated 0. 5 h after test compound administration. The reaction time was scored when the animal jumped or licked its paws. A maximum latency (cutoff) was set at 30 s to avoid tissue damage. The other two groups of mice were selected, and the experiment was repeated at 52 °C (± 0.5 °C) and 55 °C (± 0.5 °C) under the same experimental conditions [16,20].

2.3.2. Hot water immersion tail flick test

The temperature of the water bath pot was set to 48 °C (± 0.5 °C) and the mice were placed in the container with their tails hanging naturally. Results of tail flick latencies were expressed in terms of reaction time in seconds. The latency was evaluated after MJS administration 0.5 h. The normal latency (reaction time) was approximately 15 s. A cut off time of 30 s was selected in order to avoid any tissue damage in mice. The other two groups of mice were selected, and the experiment was repeated at 50 °C (± 0.5 °C) under the same experimental conditions [21].

2.3.3. Writhing response test

Glacial acetic acid (0.8 % v/v, diluted in saline, 10 ml/kg) or vehicle was injected into the peritoneal cavities of mice. Each mouse was placed in a large disc, and the intensity of nociceptive behavior was quantified by counting the total number of writhes occurring between 0 and 10 min after stimulus injection. The writhing response of mice consisted of a contraction of the abdominal muscle together with a stretching of the hind limbs [16].

2.3.4. Formalin test

The time spent on licking and flinching the right hind paws of mice were determined between 0 and 30 min after intraplantar injection of 10 μl of formalin 5 %. Results were obtained for both the first (0–5 min) and second (10–30 min) phases [16,20].

2.4. Scratching model

2.4.1. Neck models of acute itch

The back of the neck of mice were shaved 2 days before experiments. Prior to the experiment, mice were placed in specific plastic chambers (10 cm × 10 cm × 13 cm) to adapt for 30 min. Then, mice were removed and treated with MJS. After that, mice were put back into the chambers. Thirty minutes later, mice were intradermally injected with 50 μl of histamine (200 μg/, Sigma), C48/80 (100 μg, Sigma), Chloroquine (CQ, 200 μg, Sigma) and BAM8-22 (50 μg, Sigma) in the back of the neck separately. Scratching behavior of mice was video recorded (Nikon, Japan) and assessed blindly. Hindlimb scratching behavior toward the injected area of the neck was observed for 30 min with 5-min intervals [1,11,22].

2.4.2. Dry skin-induced chronic itch model in the neck

Dry skin pruritus model was made using a previously reported AEW treatment method with minor modifications [23,24]. All mice were shaved at the neck injection area 2 days before experiments. The back of nape was painted with mixture of acetone and diethyl ether (1:1) for 15 s, immediately followed by treatment with distilled water for 30 s, twice daily (at 10 A.M. and 5 P.M.) for 7 consecutive days. Mice were treated with MJS (7.5 mg/kg) 0.5 h before dry skin process started from the 4th to the 7th day of modeling. The spontaneous scratching was video recorded for 1 h for continual 7 days before dry skin process started or MJS injection and scratches were counted blindly. In addition to the MJS intervention group, two positive control drugs were also used, a H_1 receptor antagonist chlorpheniramine (CLP, 400 mg/kg) and a TRPV4 antagonist HC067047 (250 ng/kg). Three intervention drugs of mice were administered by intraperitoneal injection respectively.

2.5. Cheek models of acute pain and itch

Mouse cheek was shaved 2 days before experiments. Mice received an intradermal injection of 10 μl GSK101 (10 μg, Sigma) into the cheek and counted the number of wipes and the number of scratches for 30

min. The unilateral wipes with the forelimb of mice were counted. The scratches were defined as a lifting of the hind paw of mice toward the injection site on the cheek and then returning the paw to the floor or to the mouth [25–27].

2.6. Hematoxylin-eosin (HE) staining for histopathology of skin tissues

Mice were terminally anesthetized with isoflurane and perfused with ice-cold normal saline and 4 % paraformaldehyde. The skin of the mouse neck and back was collected and postfixed in 4 % paraformaldehyde overnight before being dehydrated, embedded in paraffin and serially sectioned (4 μm) for histological analysis. The paraffin sections were stained with hematoxylin and eosin, dehydrated through a graded series of ethyl alcohol and xylene. skin tissue were identified under magnification (× 200) light microscope (Nikon eclipse 80i microscope L, Japan) using the Imaging-Pro-Plus 6.0 software. Each sample randomly selected 5 fields of view for observation.

2.7. Immunofluorescence staining

After the perfusion, the dorsal root ganglion (DRG) of L2-L3 segment of mice corresponding to the skin at the modeling site was taken and fixed in 4 % tissue fixative for 24 h. After embedding and slicing the tissue, immunofluorescence single staining was carried out to evaluate TRPV4 distribution in the DRG neurons. The sections were deparaffinized, air-dried, and nonspecific blocking and staining were performed as described previously. Sections were incubated overnight at 4 °C with the following primary antibodies, mouse monoclonal anti-TRPV4 (1:200, Abcam, ab-39260). The sections were rinsed with phosphate-buffered saline and incubated for 1 h with secondary goat antibodies conjugated with gost anti-mouse IgG-FITC (1:400, Servicebio, GB25301) and CY3 (1:300, Servicebio, GB21303). Then, sections were counterstained with DAPI (Beyotime, 1:400, Shanghai, China). Immunofluorescence images were captured using a Laser scanning confocal microscope (Leica, Frankfurt, Germany). The mean density of TRPV4 was used to quantify its expression by the Image-Pro plus 6.0 analysis system (Media Cybernetics, Silver Spring, MD, USA), in corresponding sections of five mice in each group [28,29].

2.8. Statistical analysis

Data values are expressed as the mean ± standard error of the mean (SEM) of all independent experiments. Statistical analysis was conducted using GraphPad Prism version 8.0 (GraphPad Software, Inc., La Jolla, CA, USA). The student's t test was used to compare the means between two groups. For multiple-group comparisons, one-way variance analysis (ANOVA) with Bonferroni's multiple comparison tests was employed. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Antinociceptive effect of MJS in mice

Four types of nociception tests were performed to evaluate the analgesic effects of MJS in mice. The results are shown in Fig. 1. In the hot plate (Fig. 1A) and thermal infiltration tail flick (Fig. 1B) tests, MJS (7.5 mg/kg, i.v.) significantly prolonged the pain latencies of mice at 48, 52, and 56 °C (*$P < 0.05$, **$P < 0.01$) and at 48, 50, and 52 °C (*$P < 0.05$), respectively, compared to those in saline-treated mice. Correspondingly, MJS (7.5 mg/kg, i.v.) significantly reduced the total twisting number (Fig. 1C) in the glacial acetic acid twisting test (***$P < 0.001$) and shortened the duration of licking and flinching (Fig. 1D) in the inflammatory pain experiment caused by formalin compared with the control values (*$P < 0.05$, **$P < 0.01$).

3.2. Anti-pruritus effect of MJS in histamine-dependent and -independent itch

We intradermally injected with histamine (200 μg/50 μl; Sigma) and C48/80 (100 μg/50 μl; Sigma) to observe the anti-pruritus effect of MJS in acute histamine-dependent itch. As shown in Fig. 2, MJS (7.5 mg/kg, i.v.) administration inhibited the pruritus response induced by

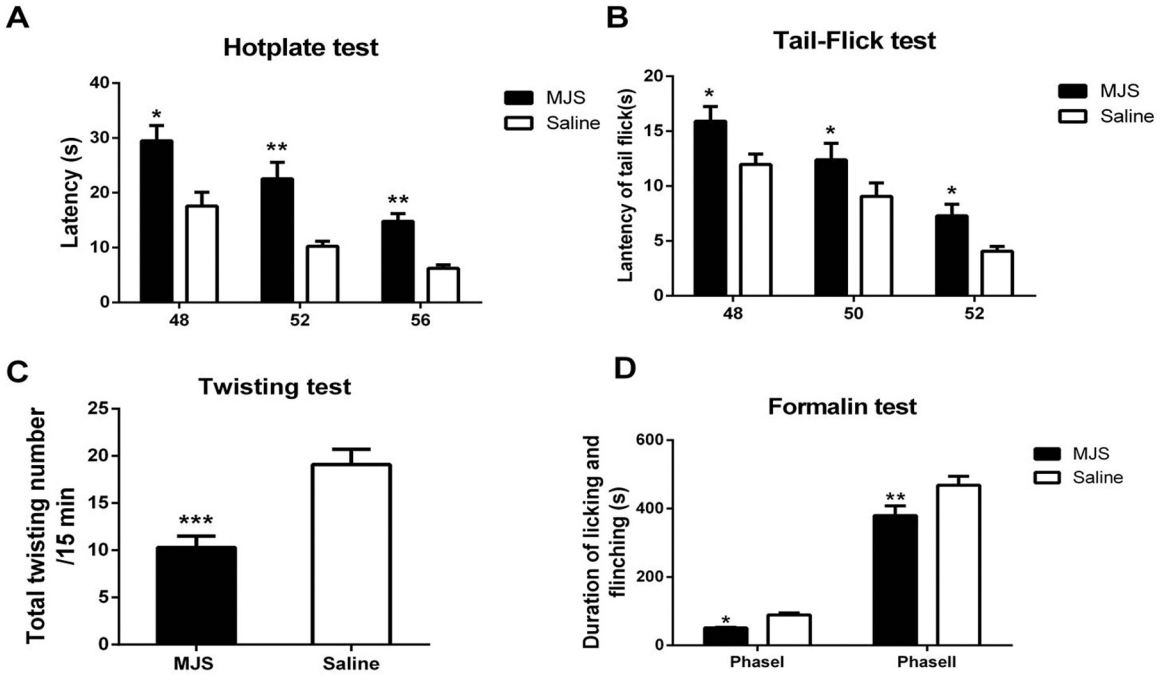


Fig. 1. Antinociceptive effect of MJS in mice. Four kind of nociception tests were used to evaluation the antinociceptive effect. (A) Hot plate test, selecting 48, 52 and 55 °C for thermal stimulation; (B) Hot water immersion tail flick test, selecting 48, 50 and 52 °C for thermal stimulation; (C) Glacial acetic acid writhing response test; (D) Formalin test. Mice were treated with MJS (7.5 mg/kg, i.v.) 0.5 h before test. Data were expressed as mean ± SEM, n = 10 in each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, student's t test when compared to the vehicle treated group.

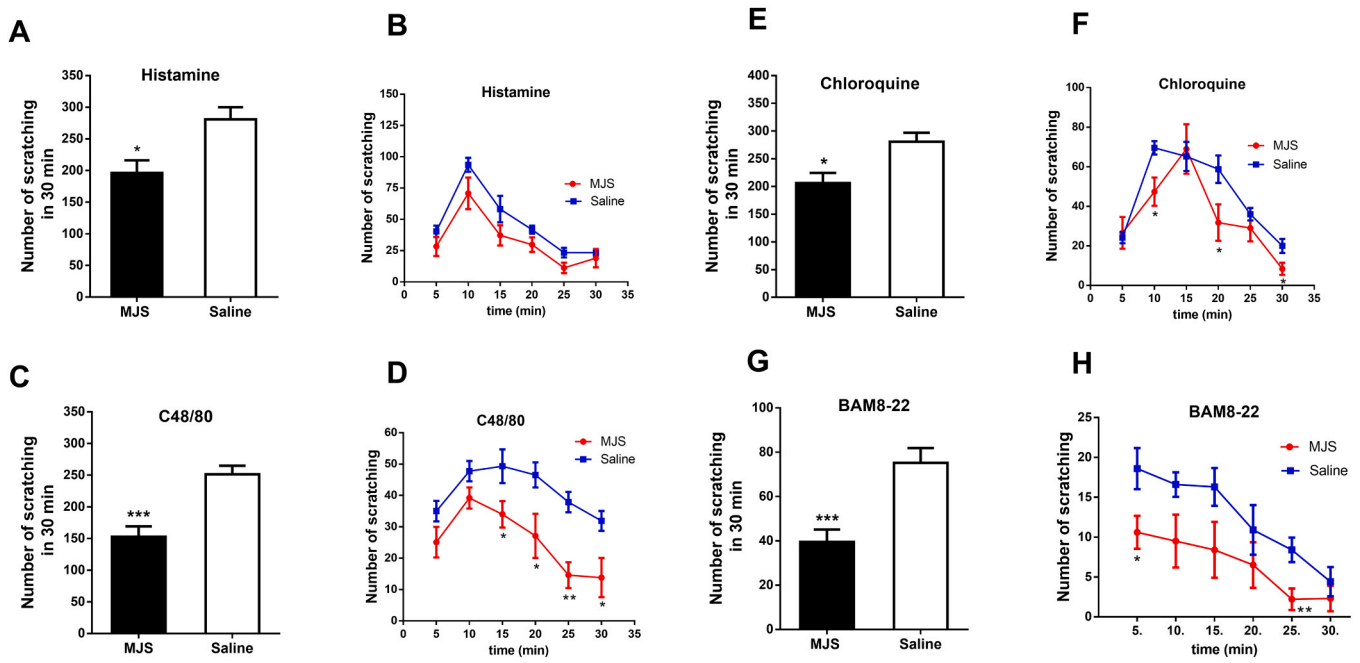


Fig. 2. Anti-pruritus effect of MJS in histamine-dependent and -independent itch. Four kinds of pruritogens were intradermally injected into the back of the neck separately, respectively are histamine 200 μg/50 μl (A, B); C48/80 100 μg/50 μl (C, D); CQ 200 μg/50 μl; (E, F) and BAM8-22 50 μg/50 μl (G, H). Scratching response in 5-minute interval and total number of scratching were recorded for 30 min. Mice were treated with MJS (7.5 mg/kg, i.v.) 0.5 h before pruritogens injection. n = 10 in each group. Data were expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, student's *t* test (total number of scratch bouts) and Bonferroni's multiple comparison tests (scratching in bouts/min) when compared to the vehicle treated group.

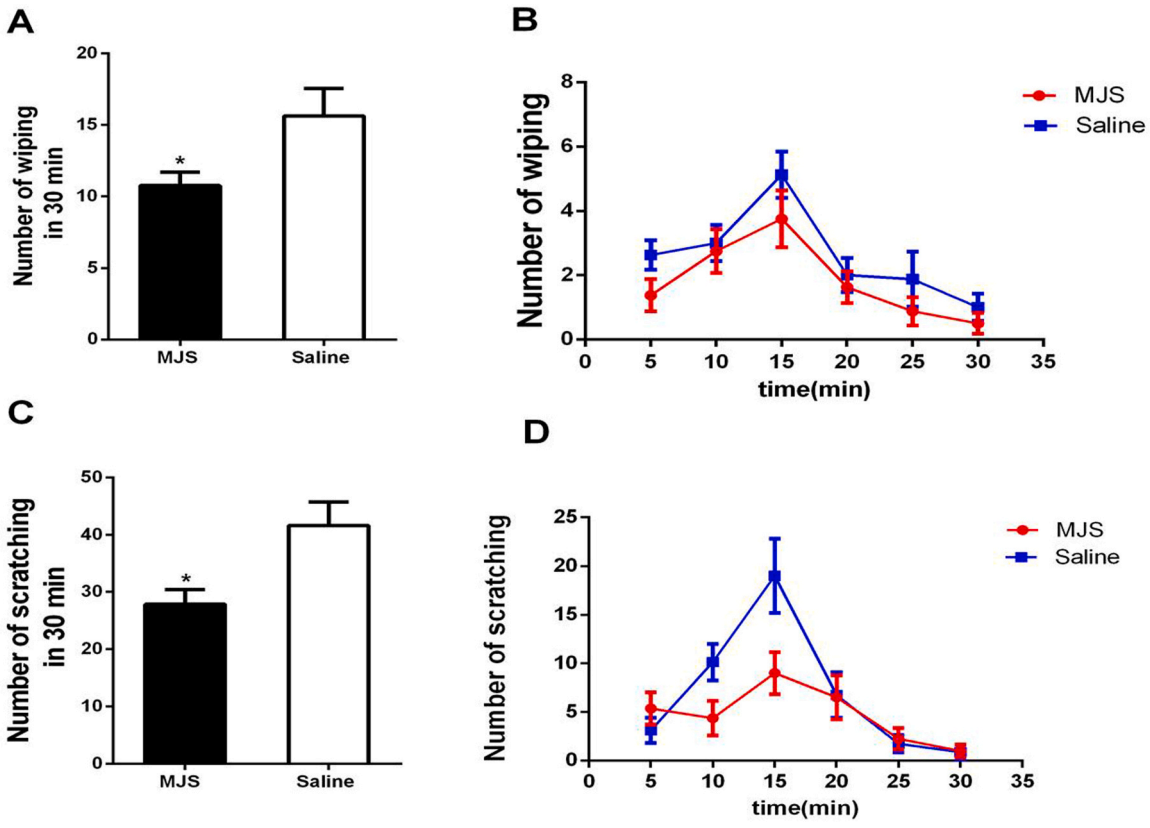


Fig. 3. Inhibitory effect of MJS on pain and itching induced by TRPV4 pathway activation. Cheek models were made by injection GSK101 (10 μg/5 μl) to elicit pain and itch behavior. (A, B) forelimb wiping; (C, D) hind limb scratching. Mice were treated with MJS (7.5 mg/kg,i.v) 0.5 h before GSK101 injection. n = 10 in each group. Data were expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, student's *t* test (total number of scratch bouts) and Bonferroni's multiple comparison tests (scratching in bouts/min) when compared to the vehicle treated group.

histamine (Fig. 2A, B) and C48/80 (Fig. 2C, D) in mice. The total number of scratch bouts (Fig. 2A, C) and the temporal profile of scratching in bouts/min (Fig. 2B, D) rapidly decreased to baseline within 30 min compared to the control group (*$P < 0.05$, ***$P < 0.01$).

To study the inhibition of MJS in acute histamine-independent itch, we intradermally administered mice with 50 μl of CQ (200 μg) and BAM8-22 (50 μg) separately. The results were shown in Fig. 2E–H. MJS (7.5 mg/kg, i.v.) administration distinctly decreased the total number of scratching bouts (Fig. 2 E, G) and the scratching bouts/min for 30 min (Fig. 2 F, H) compared to those in the control group (*$P < 0.05$, ***$P < 0.01$).

3.3. Inhibitory effect of MJS on pain and itching induced by TRPV4 pathway activation

When specific TRPV4 agonist GSK101 (10 μg/5 μl) was injected into the cheek, the mice behavior that was completely different from that elicited when applied to the nape of the neck. Two behaviors, hind limb scratching (Fig. 3 C, D) and forelimb wiping (Fig. 3 A, B), were observed, and both reached a peak within 15 min. The scratching bouts rapidly returned to near-baseline levels within 25 min (Fig. 3D), whereas wiping lasted for 30 min and persisted (Fig. 3B). In comparison to the responses to the vehicle, MJS (7.5 mg/kg) repressed the wiping reaction of mice (*$P < 0.05$) and inhibited the total number of pruritus and scratching bouts (*$P < 0.05$) relative to the control animals.

3.4. Anti-pruritus effect of MJS on dry skin-induced chronic itch in mice

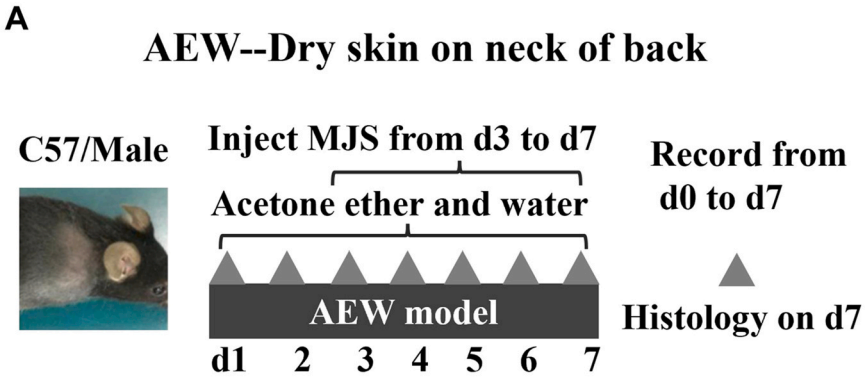
We treated mice with acetone–ether–water (AEW) on their neck skin for 7 d to mimic the symptoms of dry skin. Wild-type mice showed robust spontaneous scratching with the extension of the modeling time (Fig. 4). From days 4 to 7 of modeling, compared with the AEW model, MJS (7.5 mg/kg) drastically decreased the scratching behavior of mice induced by AEW treatment within 60 min (**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). CLP (400 mg/kg) and HC067047 (250 ng/kg) had effects similar to those of MJS in AEW mice (***$P < 0.001$, ***$P < 0.001$, ***$P < 0.001$).

3.5. Effect of MJS on chronic itch-associated cutaneous barrier disruption in mice

HE staining was used to observe the effect of MJS on histopathological changes in the skin on the back of the neck, which characterizes the itch-associated response following cutaneous barrier disruption. HE staining clearly revealed apparent epidermal hyperplasia in the AEW group compared to normal skin in the blank group (***$P < 0.001$). AEW mice showed parakeratosis, abnormally accentuated keratinization, and infiltration of inflammatory cells into the dermis (Fig. 5 B). Compared with the AEW model, MJS (7.5 mg/kg) markedly reduced the epidermal thickness of the mouse skin and alleviated inflammatory infiltration of the skin epidermis and expansion of dermal epidermal capillaries in AEW mice (Fig. 5 E). CLP (400 mg/kg) and HC067047 (250 ng/kg) had the same effect and produced a thinner epidermis than that in the model control group (***$P < 0.001$, **$P < 0.001$).

3.6. Effects of MJS on TRPV4 expression in DRG neurons and in AEW mice

We performed immunofluorescence to investigate the effects of MJS on TRPV4 expression in DRG neurons using a laser confocal microscope



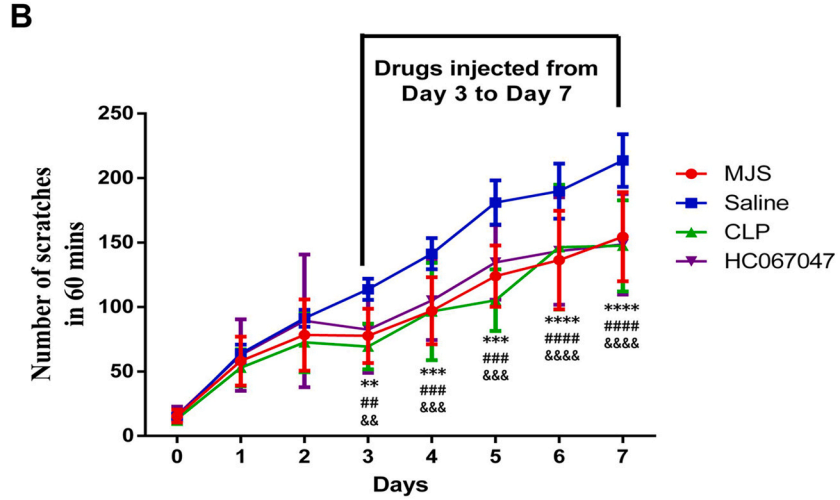


Fig. 4. Anti-pruritus effect of MJS on dry skin-induced chronic itch in mice. Mice had treated with water or AEW for 7 days. Scratching behaviors were recorded in modeling process for 8 days (d0–d7). Scratching were recorded for 1 h before dry skin process started (the first 2 days) or MJS injection (next 5 days). Mice were treated with MJS (7.5 mg/kg), CLP (400 mg/kg) and HC067047 (250 ng/kg) 0.5 h before dry skin process started from the 3th to the 7th day by intraperitoneal injection respectively. n = 10 in each group. Data were expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ***$p < 0.0001$, MJS vs. the AEW model. $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001, $^{\#\#\#\#}$P < 0.0001 CLP vs. the AEW model. $^{\&\&}$P < 0.01, $^{\&\&\&}$P < 0.001, $^{\&\&\&\&}$P < 0.0001 HC067047 vs. the AEW model. Bonferroni's multiple comparison tests when compared to the AEW model.

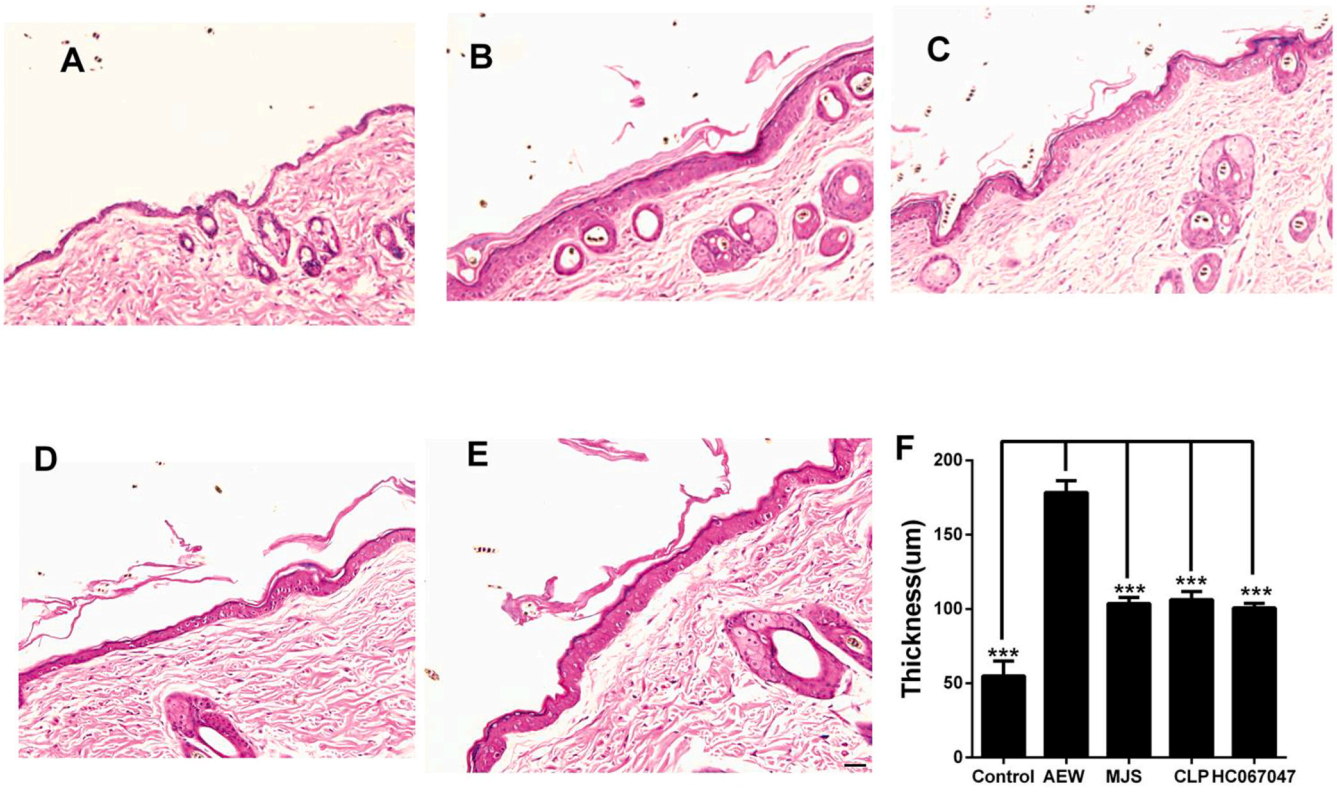


Fig. 5. Effect of MJS on chronic itch-associated cutaneous barrier disruption in mice. Micrographic images (× 200) of H&E-stained back of neek skin, mice had treated with water or AEW for 7 days. (A)sham; (B) AEW model; (C) CLP; (D) HC067047; (E) MJS; (F) The thickness of nucleated epidermal layers was quantified for 5 groups. Mice were treated with MJS (7.5 mg/kg), CLP (400 mg/kg) and HC067047 (250 ng/kg) 0.5 h before dry skin process started from the 3th to the 7th day by intraperitoneal injection respectively. Scale bar = 100 μm. n = 5 in each group. Data were expressed as mean ± SEM. ***$p < 0.001$, Bonferroni's multiple comparison tests when compared to the AEW model.

(× 800). Compared with the blank control group, AEW treatment significantly stimulated the expression of TRPV4 (Fig. 6 A, B) in DRG neurons at L2-L3 in the AEW model (**$P < 0.01$). TRPV4 expression in DRG neurons was not significantly different for MJS (7.5 mg/kg), CLP (400 mg/kg), and HC067047 (250 ng/kg) interventions compared to the AEW model.

4. Discussion

Itching and pain are distinctive unpleasant sensations triggered by the same receptive fields in the skin. Recent data suggest that there is a complex interaction between pain and itching and comparable mechanisms of neuronal sensitization [9,30,31]. Pain and itching can be triggered simultaneously and the TRP pathway can be used as a signal transmission pathway to mediate pain and itching. The TRP pathway consists of molecular receptors that respond to mechanical, chemical, and thermal stimuli, and TRPV4 plays an important role in the transmission of pain and itching [32]. Neuronal-TRPV4 may play a specific role in various types of pain as well as certain forms of acute and chronic itching [1,6,32].

The TRPV4 pathway is a non-selective cationic pathway that plays an important role in various pain responses. Clinical research has found that the TRPV4 c 0.806G > A mutation in clinical patients may be related to congenital spinal muscular atrophy with pain and loss of subjective sensation [33]. The TRPV4 pathway plays a crucial role in sensing acidic solutions, heat sources, and in the development of pain in Freund's adjuvant-inflamed pain model. Protein kinase C activates the TRPV4 pathway through phosphorylation [8,34]. TRPV4 expression is upregulated in inflammatory pain, and pain can be alleviated in *Trpv4* conditional knockout mice [35]. In contrast, the TRPV4 agonist GSK-101 increases mechanical and thermal hyperalgesia in rats with neuropathic pain [36]. MJS is a C-glycosyl flavonoid with potent analgesic effects. Its mechanism may be related to the morphine receptor antagonism and its anti-inflammatory effect, whereas others have speculated that it may be related to the antagonism of TRPV1 [16–19]. In this study, we observed the antinociceptive effect of MJS on injury risk stimulation and found that MJS had a strong inhibitory effect on heat, chemical and inflammatory pain stimulation, which is consistent with previous reports. According to experimental results, MJS notably inhibited thermal infiltration, chemical stimulation, and inflammatory pain. Whether the analgesic effect of MJS is related to the inhibition of the TRPV4 pathway warrants further study.

In light of the close correlation between itching and pain, we generated classic acute itching models, including histamine-dependent and -independent models, to investigate the potential effects of MJS on acute itching, and achieved significant results. Both of these kinds of models are involved in the TRPV4 pathway [11,12]. Histamine is the best-known cause of endogenous itching. The TRPV4 pathway is involved in histamine-induced pruritus and TRPV4 knockout mice exhibit significantly reduced sensitivity to this pathway [1,11]. CQ causes itching in the absence of histamine by activating MrgprA3. TRPV4 knockout mice with CQ-induced pruritus have been reported to show significantly reduced pruritus [37]. Our results showed MJS (7.5 mg/kg, i.v.) significantly reduced histamine-, C48/80-, CQ-, and BAM8-22-induced pruritus. The data showed that MJS had a remarkable antipruritic effect on acute histamine-dependent and -independent itching. Whether the antipruritic effect of MJS is related to the inhibition of the TRPV4 pathway also warrants further research.

Physicians often prescribe analgesics to patients with itching, suggesting common mechanisms underlying pain and itching, especially peripheral and central sensitization [38]. Our results showed that MJS has both analgesic and anti-itching effects and that TRPV4 is involved in

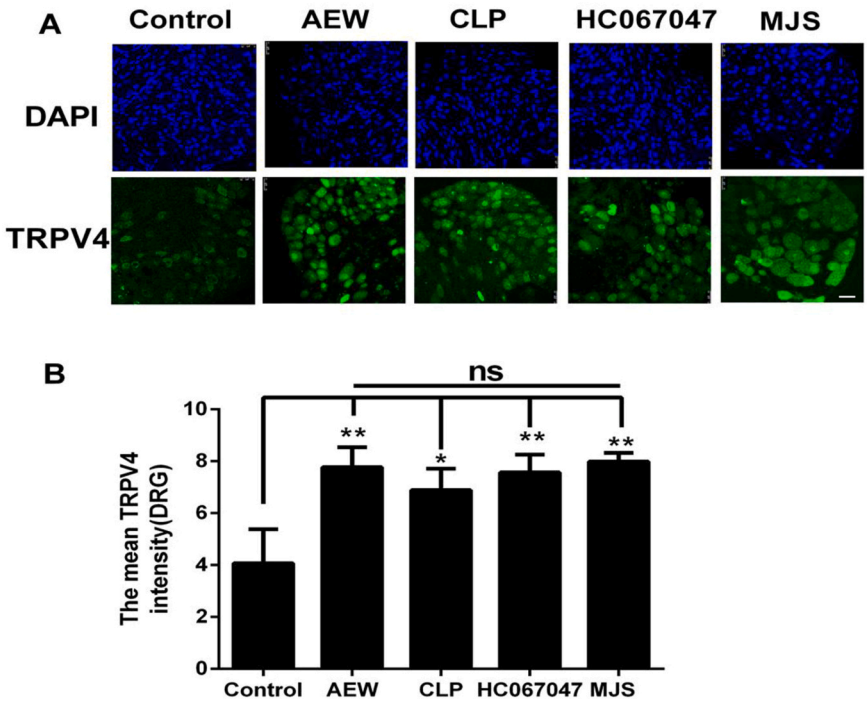


Fig. 6. Effect of MJS on TRPV4 expression in DRG neurons in AEW mice. (A) Immunofluorescence results for TRPV4 expression of DRG neurons in AEW mice, micrographic images (× 800); (B) The mean intensity of TRPV4 in DRG; n = 5 in each group. Scale bar = 50 μm. Data were expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, Bonferroni's multiple comparison tests when compared to the vehicle treated group. NS means there was no statistical difference in each drug group; $p > 0.05$, Bonferroni's multiple comparison tests when compared to the AEW model.

itching and pain simultaneously [31]. Therefore, it is necessary to explore whether the effects of MJS are related to the TRPV4 pathway. We used a special TRPV4 agonist, GSK 101, preparing a cheek model to explore the inhibitory effect of MJS on TRPV4. The mouse cheek model provided a behavioral differentiation of chemicals that elicit scratching with the hind limb as an indicator of itch, and wiping with the forelimb as an indicator of pain [25]. Our results showed that demonstrating MJS significantly reduced the occurrences of wiping and scratching evoked by GSK 101, a good inhibitory effect on pain and itching. This effect may be related to the regulation of the TRPV4 pathway.

We further developed an AEW dry skin model to observe the antipruritic effect of MJS on chronic itching in mice. TRPV4 plays a pivotal role in dry skin-induced pruritus, and an antagonist of TRPV4, HC067047, ameliorates dry skin conditions in AEW mice. MJS drastically decreased spontaneous scratching behaviors in AEW mice, markedly reduced the epidermal thickness, and alleviated the inflammatory infiltration of the skin epidermis and the expansion of dermal epidermal capillaries in AEW mice. We further studied the expression of TRPV4 in AEW mice by immunofluorescence and western blotting, and we observed that it was significantly increased in model mice, indicating that TRPV4 was closely related to pruritus in dry skin mice. However, our results showed that MJS did not inhibit the expression of TRPV4, and although a downward trend was observed, the difference was not significant. Sensory receptor/ion channel sensitization plays an important role in the induction and persistence of sensory disorders [9]. Therefore, we speculate that MJS plays a role in the functional inhibition of TRPV4 receptors. In summary, MJS significantly inhibited pain and itching behavior, as well as itching may be caused by TRPV4 activation. For the first time, an inhibitory effect of MJS on itching was observed indicating its favorable application prospects. We will further explore the mechanism of MJS on nociceptive sensation and pruritus.

CRediT authorship contribution statement

Zhiqiang Qin: Methodology, Investigation, Data curation. **Lan Xiang:** Methodology, Investigation. **Siyu Zheng:** Methodology, Visualization. **Yuchen Zhao:** Statistical analysis, Writing – review & editing. **Yanyan Qin:** Methodology. **Lei Zhang:** Visualization. **Lanlan Zhou:** Conceptualization, Writing – review & editing.

Conflict of Interest

1) None of the material in the manuscript has been published previously, is included in another manuscript, or is currently under consideration for publication elsewhere.
2) The Article has not been accepted for publication elsewhere, nor have I assigned any right or interest in the article to any third party.

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Data Availability

Data will be made available on request.

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