ORIGINAL ARTICLE

Charantin Relieves Pain by Inhibiting Pro-Inflammatory Cytokine Induction

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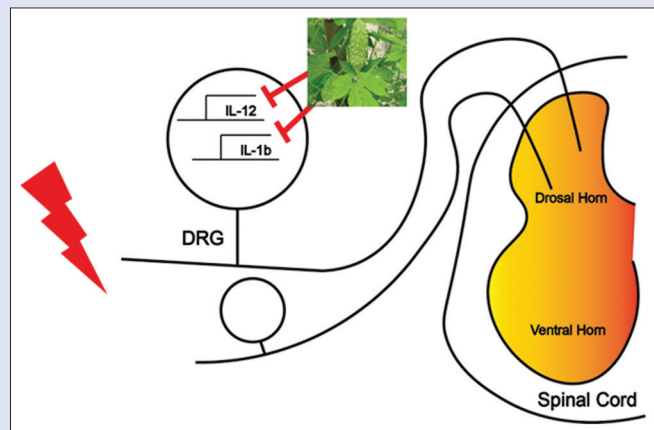
ABSTRACT

Background: The fruits of *Momordica charantia*, commonly known as bitter melon, have been used as a traditional medicine in several countries. Some studies have reported its pharmacological effects in various disorders. **Objectives:** Because there have been little reports on charantin's role as an analgesic, we evaluated its pain relief effect to determine if it could be a novel pain killer candidate. **Materials and Methods:** We established post-operative and neuropathic pain models, which represent acute and chronic pain, respectively. Mechanical withdrawal threshold assay and ultrasonic vocalization analysis were used as behavioral tests. **Results:** The administration of charantin reduced both the post-operative and neuropathic pain. The application of charantin did not make a difference in the activation of action potentials of dorsal root ganglion (DRG) neurons. However, charantin inhibited the induction of the pro-inflammatory cytokines interleukin IL-12 and IL-1β in DRG neurons. **Conclusion:** Our findings indicate that charantin seems to relieve pain by inhibiting the inflammatory process rather than by directly influencing the activity of neurons. We conclude that charantin, the commercially available extract from *M. charantia*, has great efficacy as a novel analgesic compound.

Key words: Analgesic effects, charantin, dorsal root ganglion neuron, plantar incision, pro-inflammatory cytokine, spared nerve injury

SUMMARY

- There has been little research on the analgesic role of charantin, an extract of *Momordica charantia* fruits. In this study, we revealed that charantin has great efficacy in pain relief. The charantin-treated groups were significantly alleviated in regard to plantar incision- and spared nerve injury-induced hypersensitivity by reducing pro-inflammatory cytokine levels. These results suggest that charantin could be a novel analgesic compound in both post-operative and neuropathic pain.

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INTRODUCTION

Pain is an essential sense for the survival of all living animals.[1] Pain makes animals to escape immediately from dangerous stimuli. It can help minimize the damage to individuals from trauma. However, unmanaged long-lasting pain can diminish the quality of life. Many researchers have been studying the exact mechanisms of uncontrolled pain and have been finding novel substances to lessen uncontrolled pain. Although some kinds of drugs are currently available as pain killers, repetitive treatment of them could cause several side effects.[2,3] For instance, the representatives as anti-pain drugs are nonsteroidal anti-inflammatory drugs, which could result in liver failure, and gastrointestinal lesions.[4,5] Therefore, there are emerging trials looking for novel substances for the production of safe and effective analgesics.[6,7] Recent studies have focused on natural products as possible innovative medical substances.[8-10]

Momordica charantia, commonly known as bitter melon, grows in Asia, East Africa, and Latin America. The fruits of *M. charantia* have been used as a traditional medicine in many countries,[11] such as China,

India, Peru, Brazil, and Colombia. The fruits of *M. charantia* are used to treat various disorders,[12,13] including diabetes,[14,15] gout,[16] asthma, and wounds.[17,18] Recent studies have shown scientific evidence about the medicinal properties of *M. charantia* fruits, which contain several phytochemicals and flavonoids with various pharmacological activities, such as antidiabetic, antibacterial, antiviral, anticancer, immunomodulatory, hypotensive, antioxidant potential, analgesic, and anti-inflammatory activities.[12,19] However, little is known about the capacity of *M. charantia* fruits in the alleviation of pain.

In this study, we evaluated the pain-reducing efficacy of charantin, a commercially available extract of *M. charantia*, in a post-operative rat model[20] and a neuropathic rat model.[21] In addition, we attempted to determine the mechanism whereby charantin could be reducing pain.

MATERIALS AND METHODS

Charantin: *M. charantia* L. extracts – Charantin (CAS No. 57126-62-2) was purchased from BOC Sciences (NY, USA).

Animals and treatments

All animal experiments were performed with the guidelines of the Korea Food Research Institutional Animal Care and Use Committee (KFRI-M-13003-1). Male Sprague-Dawley rats (Samtako Bio Korea, Gyeonggi-do, Korea) were adapted under a controlled temperature (24°C) and 12-h light/dark cycle for 1 week before the experiments. 2% isoflurane was used for anesthetization for the surgery. After the plantar incision (PI) or spared nerve injury (SNI) surgeries, the rats were divided into the following three groups: (1) the vehicle-treated group, (2) the naproxen-treated group, and (3) the charantin-treated group. Naproxen (0.9%, Sigma-Aldrich Co., St. Louis, MO, USA), a well-known analgesic, was used as a positive control. Charantin was administered immediately after surgery. Naproxen was supplied through intraperitoneal injection. After the SNI surgery, charantin was administered once a day for consecutive 15 days.

Plantar incision of post-operative pain model

The PI was performed as previously described.[20] In brief, after anesthetizing rats with 2% isoflurane, we performed the PI in the skin and fascia of sole, which was incised longitudinally approximately 1 cm with a scalpel. It took progress at about 0.5 cm away from the edge of the heel toward the toes. The plantar muscle was elevated and incised longitudinally. After stop bleeding with gentle pressure, the skin was sutured with polyamide monofilaments.

Mechanical withdrawal threshold analysis

Mechanical withdrawal threshold (MWT) analysis was examined as the previous description.[22] After rats were placed on a wire grid, the plantar surface of the paw was stimulated with von Frey monofilaments (Stoelting, Wood Dale, IL, USA) by ascending force with a series of monofilaments. The lowest force at which paw withdrawal behavior occurs was determined as the threshold. Three independent trials of withdrawal were recorded.

Ultrasonic vocalization call analysis

Ultrasonic vocalization (USV) measurement was performed as previously described.[22] After introducing the post-operative pain, USVs at 22–27 kHz were evaluated and counted for 10 min with Sonotrack ultrasonic microphones (Metris B. V., KA Hoofddorp, The Netherlands), which were placed at 25–30 cm far from the heads of the animals.

Spared nerve injury of the neuropathic pain model

SNI was performed as previously described.[23] To expose the sciatic nerve and its three branches, we make a small skin incision with fine scissors after shaving the skin on the lateral surface. The common peroneal and tibial nerves were injured by ligation with 5.0 silk, followed by section distal to the ligation. The skin was sutured with polyamide monofilaments. In the sham group, the sciatic nerve and its branches were exposed as in experimental groups (SNI and SNI + charantin groups), but they were neither ligated nor transected.

Primary cultures of rat dorsal root ganglion neurons

Dorsal root ganglion (DRG) cultures were prepared as previously described with slight modifications.[24] Briefly, bilateral DRGs were dissected from 2-day-old rat pups and transferred into ice-cold Hank's Balanced Salt Solution (HBSS) without Ca^{2+}/Mg^{2+} (Gibco Cat No. 14170, Grand Island, NY, USA). The DRGs were incubated with papain solution (40 U/ml, Worthington Cat No. 3126, Lakewood, NJ, USA) for 10 min and with collagenase (0.4%, Worthington Cat No. 4176, Lakewood, NJ, USA)/dispase (0.5%, Roche Diagnostics Cat No. 165859, Indianapolis, IN, USA) solution for 10 min at 37°C to obtain a single DRG cell suspension. After pipetting 5 times with 1-ml pipette tips, the DRGs were centrifuged at 1000 rpm for 3 min. The DRG pellets were resuspended in F12 medium (Gibco Cat No. 11765, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and NGF (50 ng/ml, Sigma-Aldrich Cat No. N6009, St. Louis, MO, USA) and plated onto laminin/poly-d-lysine-coated coverslips. Before calcium imaging, the DRG cells were incubated for at least 3 h.

Cytokine analysis

For measuring the expression of interleukin (IL)-12 and IL-1β from the isolated L4, L5, and L6 DRGs, we used multiplex ELISA cytokine assays according to the manufacturer's instructions (Quansys Biosciences, Logan, UT, USA; BioLegend, San Diego, CA, USA).

Intracellular calcium imaging

DRG calcium imaging was performed as previously described.[25] In brief, the DRG cells were loaded with 5 μM Fura-2/AM (Thermo Fisher Scientific Cat No. F-1221, Waltham, MA, USA) in a dark 37°C incubator for 30 min and then washed with HEPES buffer to remove unincorporated Fura-2/AM. The buffer composition was 130.0 mM NaCl, 3.0 mM KCl, 0.6 mM $MgCl_2$, 2.0 mM $CaCl_2$, 1.0 mM $NaHCO_3$, 5.0 mM glucose, and 10.0 mM HEPES (pH 7.4). Coverslips with DRGs were mounted in a perfusion chamber and perfused continuously with HEPES buffer. The DRG neurons were excited for 200 ms at 340 and 380 nm wavelengths for detection of intracellular free calcium using a DG4 rapid wavelength system. Images were captured every 5 s. The DRG neurons were depolarized with 30 mM KCl or 30 mM KCl containing charantin (100 μg/ml). A high potassium (30 mM) solution was made by equimolar exchange of NaCl with KCl. The effect of charantin on the intracellular calcium concentration was assessed by perfusion of the DRGs for 5 min with HEPES buffer containing charantin (100 μg/ml).

Statistical analysis

All data were analyzed using one-way analysis of variance, followed by Tukey's *post hoc* test. All data contained the mean and standard error mean.

RESULTS

Application of charantin yields a pain-reducing effect in a post-operative pain model

To examine the pain ameliorative effect of charantin, a commercially available extract from fruits of *M. charantia*, we assessed the MWT in von Frey experiments on rats with or without charantin. To establish a post-operative pain model, the PI of approximately 1 cm in length longitudinally was performed. MWTs were evaluated at 0, 6, and 24 h after the PI by performing von Frey experiment independently eight times. Naproxen was treated as a pain killer drug through intraperitoneal injection.

Rats in PI group, without pre- and post-operative treatment with charantin, showed a rapid decline of MWT from 53.200 ± 4.533 (g) to 0.640 ± 0.065 (g) and 0.820 ± 0.128 (g) at 6 and 24 h after the

incision, respectively. This result indicates that post-operative pain introduced by the PI was sufficient to confirm the pain response [Table 1 and Figure 1]. As expected, rats in the PI + naproxen (30 mg/kg) group showed less sensitive behavior after the incision (6 h, 5.100 ± 1.197 g; 24 h, 4.600 ± 1.188 g). Compared with the PI group, the MWTs increased in the PI + charantin group after the incision. The increase of MWT is dependent on the concentration of charantin. At 6 h after the incision, PI + charantin 30, 100, and 300 mg/kg were 2.233 ± 0.367, 2.667 ± 0.422, and 4.175 ± 0.738 g, respectively. At 24 h after the incision, PI + charantin 30, 100, and 300 mg/kg were 1.633 ± 0.174, 3.233 ± 0.880, and 4.200 ± 1.037 g, respectively. This result means that the sensitivity to pain was obviously decreased by the charantin [$P < 0.05$, Table 1 and Figure 1]. Pain relief with charantin 300 mg/kg treatment was as effective as that of naproxen. This result suggests that the application of charantin could help to relieve pain in rats.

Application of charantin reduces ultrasonic vocalization calls induced by the plantar incision

Measuring the USV calls is a valuable tool for evaluating emotion and status in rodents. When the rats feel pain, it is known that the count of USV calls at 22–27 kHz has increased.[26] After the PI, we measured the USV calls to confirm the pain relief effect of charantin. The PI group consisted of rats without administration of charantin after the PI. The frequency of USV calls in the PI group was 19.250 ± 6.175; in contrast, the number of USV calls remained low (5.875 ± 1.684) in the PI + charantin group to which charantin was administered at 6 h after the PI [Figure 2]. A similar phenomenon was also detected in groups at 24 h after the incision. Since treatment with charantin had an effect on the USV calls of rats at 22–27 kHz, we have checked the frequency of ultrasounds of rats without PI at 22–27 kHz. As expected, no ultrasounds

at 22–27 kHz were detected in nonincised rat groups with or without charantin. This result implies that the application of charantin has the effect of pain relief.

Administration of charantin relieved pain response in a rat neuropathic pain model

We evaluated whether charantin could reduce neuropathic pain as well as post-operative pain. To introduce neuropathic pain, we carried out a SNI surgery in rats. The SNI is one of the well-established methods for inducing neuropathic pain. The SNI surgery consists of axotomy

Table 1: The value of mechanical withdrawal threshold (g)

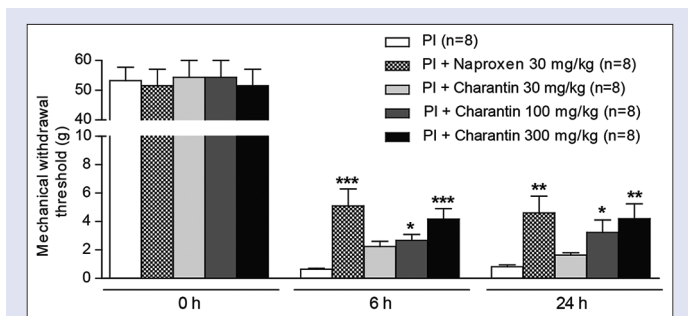|	0 h	6 h	24 h
PI	53.200±4.533	0.640±0.065	0.820±0.128
PI + naproxen (30 mg/kg)	51.500±5.565	5.100±1.197	4.600±1.188
PI + charantin (30 mg/kg)	54.333±5.667	2.233±0.367	1.633±0.174
PI + charantin (100 mg/kg)	54.333±5.667	2.667±0.422	3.233±0.880
PI + charantin (300 mg/kg)	51.500±5.565	4.175±0.738	4.200±1.037

PI: Plantar incision

Figure 1: Reducing mechanical sensitivity by application of charantin in the post-operative pain model. Administration of charantin significantly reduced the withdrawal behavior by measuring von Frey stimulation at both 6 and 24 h after surgery. Reduction of mechanical sensitivity was dependent on the dose of charantin. Naproxen (30 mg/kg, *n* = 8) was used as the pain relief control. The asterisks stand for significant difference between the plantar incision + charantin or naproxen group and the plantar incision group, *$P < 0.05$, **$P < 0.005$ and ***$P < 0.001$. Data indicate the means ± standard error mean (*n* = 8 per group)
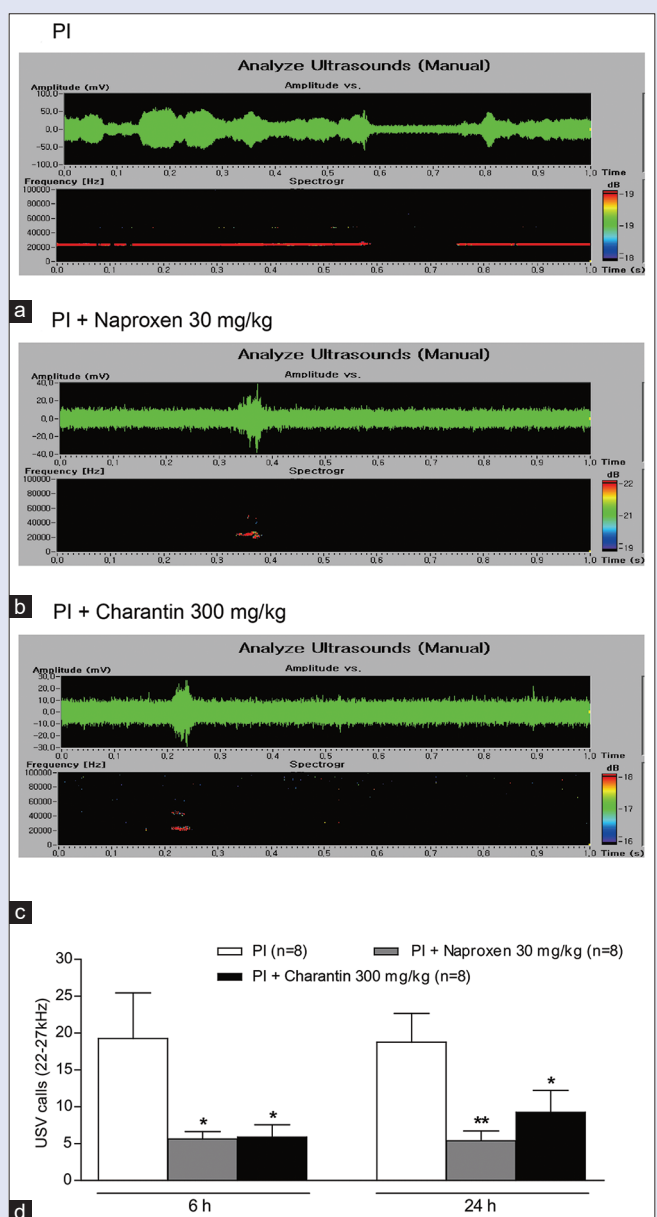

Figure 2: Application of charantin attenuated ultrasonic vocalization calls in the post-operative pain model. The sonograms of ultrasonic vocalizations from (a) plantar incision group, (b) plantar incision + naproxen group, and (c) plantar incision + charantin group rats, (d) the quantification of ultrasonic vocalization calls at 22–27 kHz. The asterisks stand for significant difference between the plantar incision + charantin or naproxen group and the plantar incision control group, *$P < 0.05$ and **$P < 0.005$. Data indicate the means ± standard error mean (*n* = 8 per group)

and ligation of two of three branches of the sciatic nerves, while the sural nerve is left intact. We then evaluated MWTs every 3 days after the procedure. As the control group, we set the sham group, in which the branches of sciatic nerve were identically exposed but neither ligated nor transected. In the sham group, MWT remained almost constant [Figure 3]. Whereas, in the SNI group, MWT showed a severe decrease, which means hypersensitivity of rats (0 day, 55.750 ± 4.250 g; 3 days, 0.850 ± 0.145 g; 6 days, 0.435 ± 0.104 g; 9 days, 0.218 ± 0.057 g; 12 days, 0.164 ± 0.038 g; 15 days, 0.098 ± 0.019 g). Naproxen was used for a pain killer as a positive control drug. The MWTs remained higher in the SNI + naproxen (0 day, 55.444 ± 4.998 g; 3 days, 4.000 ± 0.645 g; 6 days, 2.333 ± 0.446 g; 9 days, 1.756 ± 0.348 g; 12 days, 1.911 ± 0.414 g; 15 days, 1.511 ± 0.356 g) and SNI + charantin (0 day, 53.200 ± 4.533 g; 3 days, 3.860 ± 0.725 g; 6 days, 2.120 ± 0.436 g; 9 days, 1.300 ± 0.174 g; 12 days, 1.000 ± 0.193 g; 15 days, 0.796 ± 0.178 g) groups than in the SNI group, indicating that SNI + charantin group was less susceptible to neuropathic pain than SNI group. These results

Figure 3: Pain relief effect of mechanical withdrawal threshold in the spared nerve injury neuropathic pain model. Administration of charantin significantly reduced the withdrawal behavior by measuring von Frey stimulation every 3 days after spared nerve injury operation. The asterisks stand for significant difference between the spared nerve injury + charantin or naproxen group and the spared nerve injury group, *$P < 0.05$, **$P < 0.005$, and ***$P < 0.001$. Data indicate the means ± standard error mean ($n = 8$ per group)

mean that charantin is not efficient only in post-operative pain but in neuropathic pain.

Charantin did not alter the sensitization of dorsal root ganglion neurons

Because charantin reduces the sensitization to pain, we checked whether charantin could directly attenuate the activity of DGR neurons. Calcium imaging analysis was performed to detect the alteration of calcium influx with or without charantin in primary sensory neurons that were prepared from rats. Depolarization by KCl induced a calcium influx in DRG neurons, which was detected by Fura-2 dye. To select the healthy DRG neurons, the first depolarization was carried out with 30 mM KCl. Only DRG neurons that showed sufficient calcium influx were used in the second depolarization to test the effect of charantin. In control experiments ($n = 8$), as shown in Figure 4a, the difference of Fura-2 ratio after the first depolarization was 1.164, from 0.829 ± 0.047 to 1.993 ± 0.281, and by the second depolarization, it was 0.998, from 0.861 ± 0.080 to 1.859 ± 0.265. The difference of Fura-2 ratio in the second stimulation was 0.857 times smaller than in the first one. It is generally accepted that the second stimulation of neurons does not lead to a response as great as the first one.

In the charantin treatment experiments, as shown in Figure 4b, the first depolarization was performed without charantin similar to in the control experiments ($n = 7$). The difference of Fura-2 ratio after the first depolarization was 0.999, from 0.806 ± 0.102 to 1.805 ± 0.089. To test the effect of charantin, 100 µg/ml of it was applied for 200 s before the secondary depolarization. The difference after the second depolarization was 0.858, from 0.818 ± 0.108 to 1.676 ± 0.115. Similar to the control experiments, the difference of Fura-2 ratio in the second stimulation was 0.858 times smaller than in the first one. As shown in Figure 4a and b, the sensitization of DGR neurons by KCl after treatment with charantin was no different than after that without charantin. Consequently, charantin does not seem to alter the depolarization of neurons. This means that charantin works as an analgesic by other mechanisms rather than direct alteration of the activity of neurons.

Reducing expression of inflammatory cytokines by dorsal root ganglion neurons

Because charantin did not affect the activity of DRG neurons, we next investigated inflammatory effects based on some reports that neuropathic pain is related to inflammation.[27,28] To elucidate the involvement of charantin in inflammation, we checked whether the SNI procedure caused the induced expression of IL-1β and IL-12,
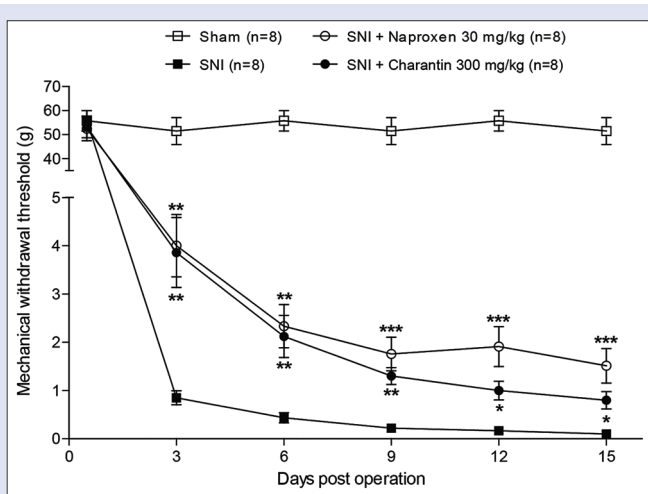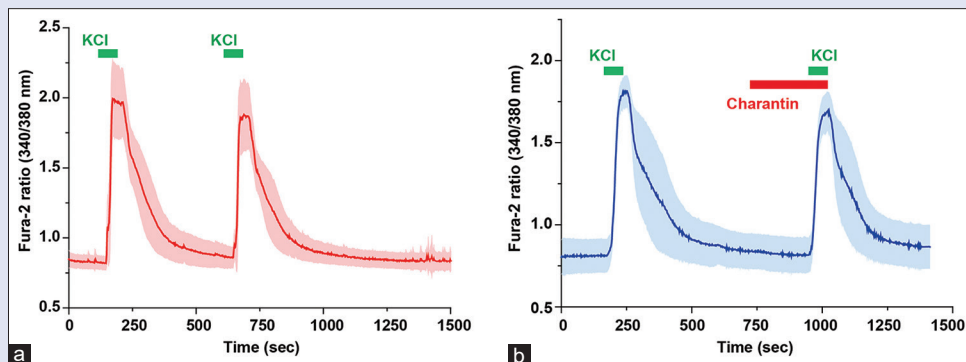

Figure 4: Charantin did not alter the sensitization of primary dorsal root ganglion neurons. Calcium imaging analysis was performed to detect the alteration of calcium influx with (b) or without (a) charantin in primary dorsal root ganglion neurons. Calcium influx was induced by depolarization using 30 mM KCl ($n = 7$) and was similar to that in control experiments (a), ($n = 8$)
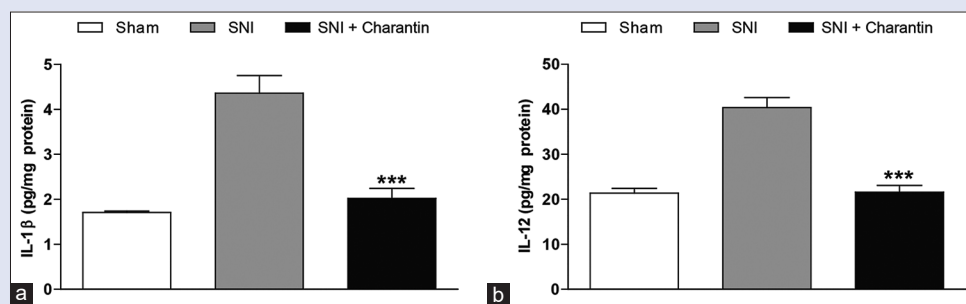
Figure 5: Application of charantin inhibits the induction of pro-inflammatory cytokines. Administration of charantin significantly inhibited the increment of expression of interleukin-1β (a) and interleukin-12 (b) in dorsal root ganglion neurons. Data are mean ± standard error mean (*n* = 8 per group). The asterisks stand for significant difference between the spared nerve injury + charantin group and the spared nerve injury group, ***$P < 0.001$

representative pro-inflammatory cytokines, from DRG neurons. The significant induction of IL-1β (4.359 ± 0.394 pg/mg protein) and IL-12 (40.391 ± 2.224 pg/mg protein) was shown at 15 days after the SNI procedure, whereas they were not induced after the sham operation (IL-1β, 1.711 ± 0.0294 pg/mg protein; IL-12, 21.406 ± 1.017 pg/mg protein). The administration of charantin inhibited an increase of expression of the pro-inflammatory cytokines in DRG neurons after the SNI procedure (IL-1β, 2.023 ± 0.220 pg/mg protein; IL-12, 21.617 ± 1.472 pg/mg protein) [Figure 5a and b]. This result suggests that charantin likely reduces pain by inhibiting the pro-inflammatory process in neurons.

DISCUSSION

In the past in some countries, the fruit of *M. charantia* has been taken as traditional medicine for various disorders.[11,29,30] Based on its usage history for various disorders, scientific evidence regarding the efficacy of *M. charantia* fruits, such as its antidiabetic efficacy, has been reported.[12,31,32] However, little is known regarding the capacity of *M. charantia* fruit administration to alleviate pain. Therefore, in the current study, we examined that charantin, a commercially available extract of *M. charantia* fruits, could be used as a novel analgesic charantin.

The behavioral responses of rats were measured by the MWT and pain-induced USV. We found that the application of charantin showed ameliorative effects against both post-operative and neuropathic pain. The post-operative pain model, which was established by an incision in the plantar surface of the hind paw, is representative of the acute pain caused by a nociceptive stimulus.[33,34] After an incision of the plantar muscle, treatment with charantin increased the MWT. We also found that treatment with charantin resulted in a reduction in the frequency of USV calls induced by post-operative pain. These results indicate that the administration of charantin leads to reduced pain sensitivity.

In addition, we wondered whether the pain-relieving efficacy of charantin was effective against chronic pain. To represent chronic pain, we established the rat spared neuropathic injury model.[21,35] Because the neuropathic pain model mimics chronic symptoms of nerve compression, the SNI model is a suitable representative of chronic pain. We found that the application of charantin attenuated hypersensitivity in the SNI rat model.

We showed that charantin could not directly alter the activation of DRG neurons. To explain the pain-reducing mechanism of charantin, we assessed the expression levels of pro-inflammatory cytokines with and without administration of charantin. In treatment with charantin group, the expression of pro-inflammatory cytokines in DRG neurons is not induced significantly. This result suggests the possibility that charantin mediates its pain-relieving effect by inhibiting the induced expression of pro-inflammatory cytokines.

It is commonly believed that inflammation is related to the pain process.[10,36] Pain is associated with tissue damage and inflammation[36,37] and is a characteristic symptom of arthritis.[38] Cytokines, inflammatory mediators, are released from injured tissue to make nerve terminal sensitizing. Some studies have revealed that DRG neurons can release pro-inflammatory cytokines as well as immune cells.[39] Pro-inflammatory IL-1β expression is induced by injuries of peripheral nerves and results in an increment of substance *P* and prostaglandin E2 in neurons.[40]

Therefore, we hypothesize that the application of charantin shows the pain-reducing effect by inhibiting the serial cascade of inflammation. Further studies are required for identification of the effectual components existing in charantin as well as the definition of inhibitory mechanism for the induction of pro-inflammatory cytokines.

CONCLUSION

The administration of charantin reduced hypersensitivity in both *in vivo* acute and chronic pain models by inhibiting the induction of the pro-inflammatory cytokines IL-12 and IL-1β in DRG neurons. Our findings provide the possibility that charantin can be used as a natural pain reliever.

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Conflicts of interest

There are no conflicts of interest.

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