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# Effect of Continuous Passive Motion Initiated After the Onset of Arthritis on Inflammation and Secondary Hyperalgesia in Rats

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**Summary**

This study investigated the effect of continuous passive motion (CPM) initiated after the onset of arthritis in rats. Rats were injected with 3 % kaolin/carrageenan in the knee joint and randomized to the control, immobilization (IM), or CPM group. The knee joints of the IM and CPM groups were immobilized with a cast for 56 days. In the CPM group, CPM exercise was administered for 60 min/day (6 times/week). Joint transverse diameter and pressure pain threshold (PPT) were assessed as indicators of inflammation, and paw withdrawal response (PWR) was assessed as indicator of secondary hyperalgesia. Central sensitization was analyzed by measuring calcitonin gene-related peptide (CGRP) expression levels in the spinal dorsal horn. In the CPM group, the PPT was significantly increased compared with the IM group from 14 to 35 days, and PWR was significantly decreased from 14 to 56 days. Additionally, CGRP expression in the super facial layer (I-II) of the spinal dorsal horn (L4-5) in the CPM group was significantly decreased compared with the IM group. Our study found the CPM initiated after the onset of arthritis promoted the recovery of inflammation and mitigated secondary hyperalgesia

**Key words**

Arthritis • Inflammation • Continuous passive motion • Immobilization • Hyperalgesia

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## Introduction

Acute pain is associated with inflammation evoked by trauma, surgical procedures, and arthritis. It is ordinarily thought that acute pain is time dependent because inflammation gradually decreases until the growth phase of tissue healing. However, in the case of severe and prolonged tissue damage, the sustained activity of primary afferent fibers induces peripheral sensitization, which increases the efficacy of synaptic transmission between primary afferent fibers and dorsal horn neurons, a process referred to as central sensitization. Severe and long-lasting noxious stimulation from peripheral tissue is a risk factor for chronic pain (Radhakrishnan *et al*. 2003).

Resting the affected side by immobilization using plaster cast or brace is useful for restoring tissue damage and is widely used for medical treatment. However, immobilization can cause muscle weakness (Booth 1977) and joint contracture (Honda *et al.* 2015). Moreover, recent



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studies have suggested that immobilization may cause hyperalgesia with central sensitization in the spinal cord (Terkelsen *et al.* 2008, Hamaue *et al.* 2013). Terkelsen *et al.* (2008) observed pain induced by transient movement and mechanical hypersensitivity in the distal forearm of human subjects after 4 weeks of cast immobilization. Hamaue *et al.* (2013) demonstrated that cast immobilization produces a time-dependent increase in mechanical hyperalgesia and that calcitonin gene-related peptide (CGRP) expression in the deeper lamina layer of the spinal dorsal horn increases in rats that have been immobilized for 8 weeks. These findings suggest that it is necessary to keep the affected limb as active as possible during the acute phase of inflammation.

Previously, it has been demonstrated that exercise has an analgesic effect and prevents development of chronic pain (Stagg *et al.* 2011, Detloff *et al.* 2014). In an experimental animal model, the use of intensive training using forced exercise wheel walking system after spinal cord injury can prevent the development of neuropathic pain (Detloff *et al.* 2014). Aerobic exercise training has been shown to reduce hyperalgesia following injury (Stagg *et al*. 2011). However, the biological effects of passive joint motion on pain have not yet been reported. Continuous passive motion (CPM) is a treatment protocol that is used to manage the ROM after joint surgery and/or treatment of the inflamed joint. CPM has biological effect on the healing and regeneration of articular tissues with better histologic properties compared to immobilization (Salter *et al.* 1981). Additionally, the expression of IL-1β and COX-2 decreases with CPM in rat meniscal chondrocytes immediately following inflammation whereas the expression of IL-10 increases (Ferretti *et al*. 2005). These results explained the molecular basis of the beneficial effect of CPM when applied during the acute phase of joint inflammation. Therefore, we hypothesized that CPM has a positive effect on the reduction of acute pain originating from inflammation and alleviating the development of secondary hyperalgesia. This study aimed to examine the effect of CPM that was initiated after the onset of arthritis on inflammation and secondary hyperalgesia in a rat model.

## Methods

*Animals*

The Ethics Review Committee for Animal Experimentation at the authors' current institution approved all experiments. Male Wistar rats (8 weeks old, Kyudo, Saga, Japan) were used for the experiments.

All rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and subsequently given a single 300-μl injection of a mixture of 3 % kaolin and 3 % carrageenan (Sigma Chemical Co, St. Louis, USA) into the right knee joint anteriorly, and then randomly divided into the following 3 groups: (1) control (n=8), (2) immobilization (IM; n=9), and (3) immobilization with CPM (CPM; n=8). The rats in the control group were untreated. All rats were housed in a 12-h light/dark cycle. Behavioral testing was usually done between 9 AM and 5 PM. Food and water were available ad libitum. All treatments mentioned below were administered under pentobarbital sodium anesthesia (40 mg/kg).

*Immobilization*

The right knee joints of rat in the IM and CPM group were immobilized in full extension, the ankle joints were immobilized in full planter flexion using a plaster cast, whereas the left leg remained free. The plaster cast was wrapped from the pelvic area to the right distal foot while their right hind limbs were kept ramrod-straight. The plaster cast was replaced at least every 2 or 3 days to prevent loosening and edema in the hind paw. The rats were able to move freely in the cage by using the three limbs that were not immobilized.

*Range of motion on knee joint flexion*

ROM was measured with a goniometer. It was defined as the angle of a straight line connecting the great trochanter and the center of the knee joint to a line connecting the center of the knee joint and the malleolus lateralis of the fibula when the knee was flexed passively under a tensile force of 0.3 N using a spring scale, as described previously (Honda *et al.* 2015).

*Continuous passive motion*

In previous studies, CPM for 60 min once a day provided better pain response in patients with a frozen shoulder (Dundar *et al*. 2009). CPM treatment for 6 days per week increased the active ROM of patient after total knee arthroplasty (Lau and Chiu 2001). In this study, CPM was applied for 60 min, once a day, 6 days a week for 56 days from the day after injection. The knee joints were flexed to an angle equivalent to the angle recorded at maximum flexion, at an angular velocity of 10 degrees/s using a mechanical ankle stretcher (Sakai Iryo, Osaka, Japan). ROM was adjusted in each

individual rat using the above-mentioned method. Following completion of daily treatment, right knee joints were re-immobilized with a plaster cast.

*Knee joint swelling*

In order to follow the changes in joint swelling over time, we measured the transverse diameter of the right knee joint using a manual caliper. Rats were placed individually in a homemade restrainer (Nakano *et al.* 2012) so that loading on the right hind limb was avoided and the knee joint was held in the maximum extended position.

*Pressure pain threshold in the knee joint*

The pressure pain threshold (PPT) of the inflamed knee joint was assessed using a Randall-Selitto apparatus (Ugo Basile, Varese, Italy). Rats were lightly restrained by hand. The rounded tip of the transducer probe (base diameter = 9 mm) was applied to the lateral side of the knee joint with linearly increasing pressure (48 g/s). The threshold was defined as the force required for eliciting the hind limb flexion reflex or vocalization. Five measurements were taken at intervals of at least 5 min, and the average of three measurements (excluding the maximum and minimum) was recorded as the PPT.

*Paw withdrawal response*

Mechanical hyperalgesia of the hind paw was tested with von Frey filaments. The animals were placed individually in a homemade restrainer mentioned above. This technique was employed because ROM limitations of the hip, knee, and ankle joint prevented the immobilized rats from placing their right hind paws on the ground. After removing the plaster cast, the rats were allowed to acclimate for 20 min before testing. The glabrous skin of the hind paw was probed 10 times using 4 g and 15 g von Frey filaments (VFFs; North Coast Medical, Morgan Hill, CA, USA) every 10 s. Lifting or pulling back of the paw or vocalization was counted as the paw withdrawal response (PWR) by a single experimenter. The 4 g and 15 g filaments were used to ascertain mechanical allodynia and hyperalgesia, respectively (Peleshok and Ribeiro-da-Silva 2011).

*Analysis of calcitonin gene-related peptide in the spinal dorsal horn*

In this study, we focused on the expression of CGRP in the spinal dorsal horn, which may play a role in central sensitization (Kangrga and Randic 1990).

At the end of the immobilization period, all the rats were anesthetized. The spinal cord (L2-3, L4-5) was removed following the transcardial perfusion of saline and 4 % paraformaldehyde dissolved in a 0.1 M phosphate-buffer (PB; pH 7.4). The tissue was soaked for 24 h in 10 % sucrose dissolved in PB, followed by 24 h in 20 % sucrose dissolved in 0.01 M phosphate-buffered saline (PBS; pH 7.4). Spinal cord frozen sections (10 μm) were cut with a cryostat. In order to inhibit endogenous peroxidases, the sections were incubated for 30 min at room temperature with 0.3 % $H_2O_2$ dissolved in methanol. Next, sections were blocked for 20 min with 5 % bovine serum albumin dissolved in PBS, followed by incubation with an anti-CGRP polyclonal antibody (1:500 rabbit; ImmunoStar Inc., Hudson, WI, USA) overnight at 4 °C. Subsequently, they were incubated with goat anti-rabbit IgG conjugated to Texas Red® (1:600, Vector labs, CA, USA) for 1 h at room temperature. Quantitative evaluation of the calcitonin gene-related peptide (CGRP) expression in the ipsilateral dorsal horn was performed using image-analysis software (NIS-Element Ver.3, Nikon Instruments Inc., NY, USA). The spinal dorsal horn was divided into the superficial (lamina I-II) and deeper layers (lamina III-VI), according to previously described criteria (Molander *et al.* 1984). The intensity of CGRP expression reflected the quantity of fluorescence observed in the superficial (lamina I-II) and deeper (lamina III-VI) layers of the spinal dorsal horn in 5 sections per tissue.

*Statistical analysis*

All data are presented as mean ± SE. Differences between groups were assessed using the one-way analysis of variance followed by Fisher's protected least significant difference *post hoc* test. Differences were considered significant at $p<0.05$.

## Results

*Changes in knee joint swelling*

The ipsilateral knee joint transverse diameter increased significantly 1 day after injection in all groups compared to baseline. There were no significant differences in the diameter among the groups at day 1 (Fig. 1). In the IM group, there were no significant differences at each testing point compared to the control group. Similarly, there was no significant difference between the CPM and control groups except at 56 days after injection.

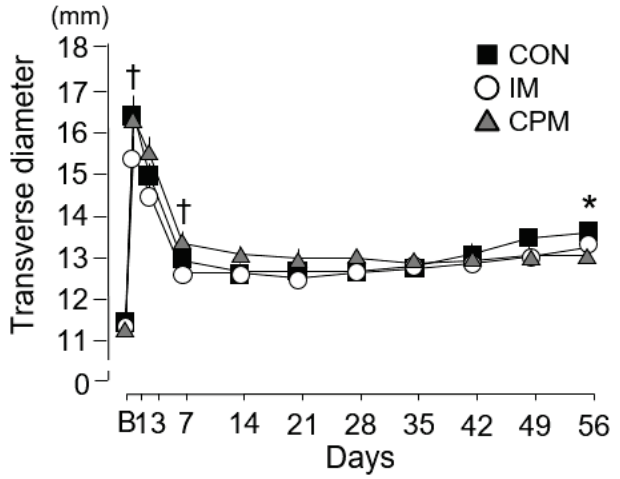


**Fig. 1.** Time course changes in the transverse diameters of the knee joints. Data are presented as the mean ± SE. * $p<0.05$ continuous passive motion (CPM) versus the control (CON) group. † $p<0.05$ immobilization (IM) versus CPM group. B, baseline.

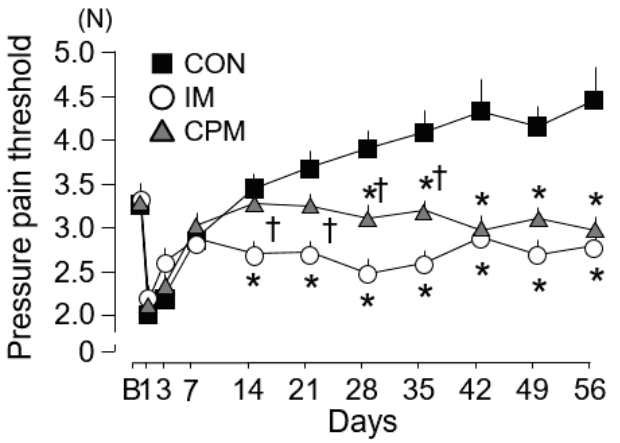


**Fig. 2.** Time course changes in the pressure pain thresholds of the knee joints. Data are presented as the mean ± SE. * $p<0.05$ continuous passive motion (CPM) or immobilization (IM) versus the control (CON) group. † $p<0.05$ IM versus CPM group. B, baseline.

*Pressure pain threshold*

The PPT 1 day after injection was significantly lower in all groups compared to baseline (Fig. 2). In the control group, the PPT was recovered 14 days after injection. The PPT in the IM group was significantly decreased compared to the control group at 14 days after injection and then remained steady until 56 days after injection. In contrast, there were no significant differences between the control and CPM groups until 21 days after injection, at which point a significant increase was found compared with the IM group from 14 to 35 days after injection. However, the PPT in the CPM group significantly decreased compared with the control group on or after day 28. Moreover, there were no significant differences between the IM and CPM groups on or after day 42.

*Paw withdrawal threshold*

The PWR of all rats, as measured by 4 g von Frey filaments for mechanical allodynia, significantly increased on the 1 day after injection compared to the baseline (Fig. 3A). In the IM group, a significant increase in the PWR was found from 21 to 56 days after injection compared to the control group. In contrast, the PWR in the CPM group was not significantly different from that of the control group, except at 21 and 42 days after injection. As measured by 15 g von Frey filaments for mechanical hyperalgesia, a remarkable increase in the PWR was identified in the IM group at 14 days after injection, which persisted for 42 days (Fig. 3B). The PWR of the CPM group was significantly decreased 14 to 56 days after injection, and no significant difference was confirmed for 35 days following injection, when compared to the control group.

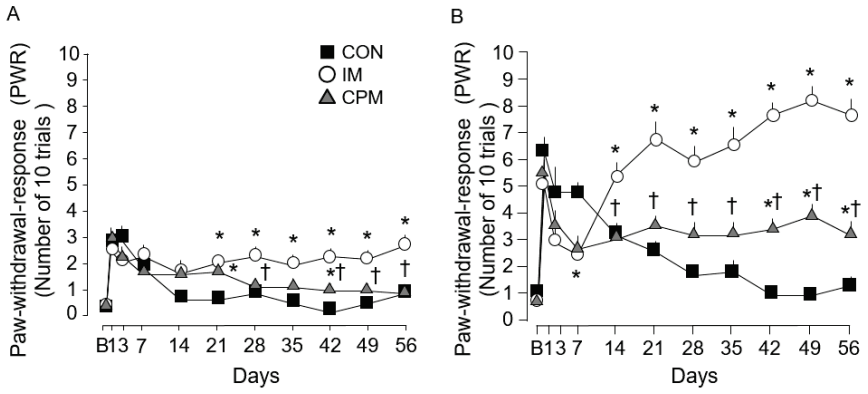


**Fig. 3.** Time course changes in the paw withdrawal thresholds of the ipsilateral hind paws. (**A**) 4 g von Frey filament (VFF) as a measurement of mechanical allodynia. (**B**) 15 g VFF as a measurement of mechanical hyperalgesia. Data are presented as the mean ± SE. * $p<0.05$ continuous passive motion (CPM) versus the control (CON) group. † $p<0.05$ immobilization (IM) versus CPM group. B, baseline.

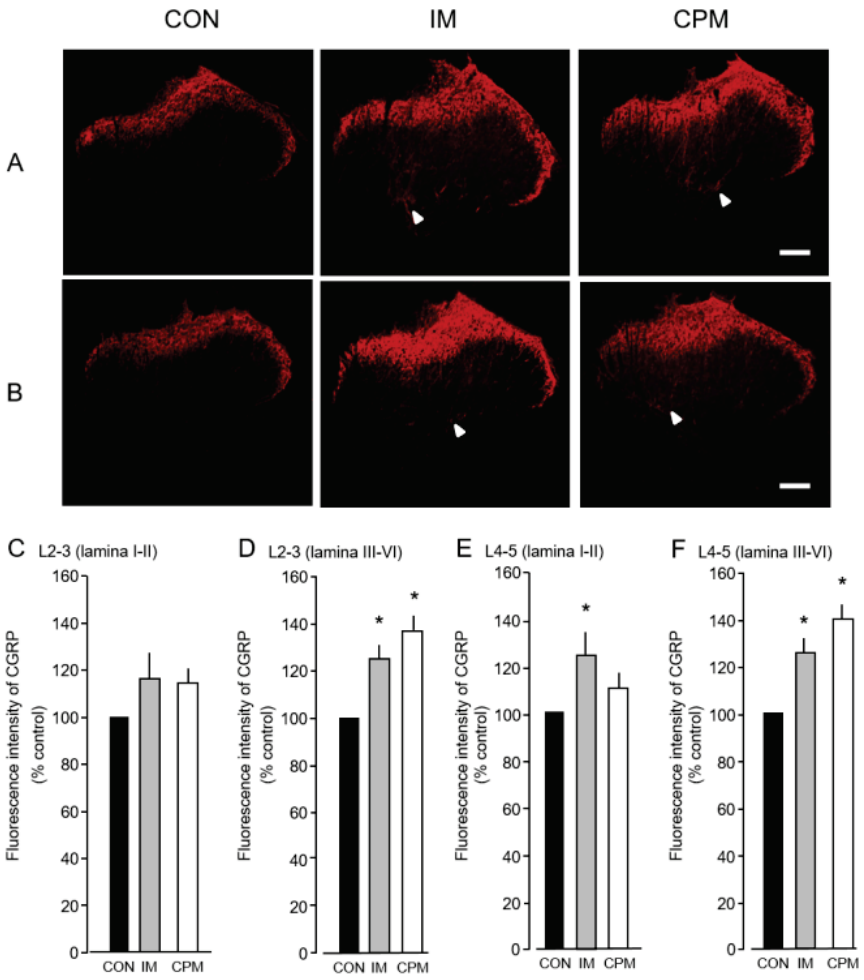


**Fig. 4.** Intensity of calcitonin gene-related peptide (CGRP) expression in the ipsilateral dorsal horn of the spinal cord. Representative photomicrographs of CGRP immunohistochemistry in the ipsilateral dorsal horn are shown, at the L2-3 (**A**) and L4-5 levels (**B**). The CGRP-positive neural fibers were clearly observed in the deep layer of the dorsal horn in the immobilization (IM) and continuous passive motion (CPM) groups (arrowheads). Percentage control of fluorescence intensity of CGRP expression in the superficial (laminae I-II) (**C**, **E**) and deep layers (laminae III-VI) were calculated (**D**, **F**) in the L2-3 (**C**, **D**) and L4-5 (**E**, **F**). Data are presented as the mean ± SE. * $p<0.05$ versus the control (CON) group. Scale bars = 100 μm

*Expression of CGRP in the spinal dorsal horn*

The CGRP immune response in the deep layer of the ipsilateral dorsal horn (L2-3) was greater in the IM and CPM groups compared to the control group (Fig. 4A). CGRP expression intensity analysis revealed no significant differences among the three groups in the superficial layer (laminae I-II) (Fig. 4C). Although CGRP expression intensity was significantly higher in the IM and CPM groups compared to the control group in the deep layer (laminae III-VI) (Fig. 4D), there were no significant differences between these two groups. In the superficial layer of the ipsilateral dorsal horn (L4-5), the CGRP expression intensity of the IM group was significantly higher than that of the control group (Fig. 4B). However, there were no significant differences between the control and CPM groups (Fig. 4E). Although the intensity of the IM and the CPM groups was significantly higher than that of the control group in the deep layer of the dorsal horn, no significant differences were noted between the IM and the CPM groups (Fig. 4F).

*ROM on knee joint flexion*

ROM of right knee flexion was decreased in all groups on the first day after injection. In the control group, this change was transient (Fig. 5). ROM in the IM and CPM groups continued to decrease until 56 days after injection. However, ROM in the CPM group significantly increased compared to the IM group from 3 to 56 days after injection.

## Discussion

The current study examined the effects of CPM that was initiated after the onset of arthritis on inflammation and pain-related behavior in rats. In previous studies, the carrageenan model was commonly used for experimental joint inflammation (Okamoto *et al.* 1999, Radhakrishnan *et al.* 2003) because of plasma extravasation after the release of neuropeptides (Lam and Ferrell 1993) and other inflammatory mediators, such as prostaglandins (Nantel *et al.* 1999), and bradykinin (Birrell *et al.* 1993). These noxious chemicals sensitize primary afferent fibers resulting in primary and secondary

hyperalgesia (Schaible and Schmidt 1988, Radhakrishnan *et al.* 2003). Therefore, this arthritis model is favorable for the examination of the effects of CPM on inflammation and secondary hyperalgesia.

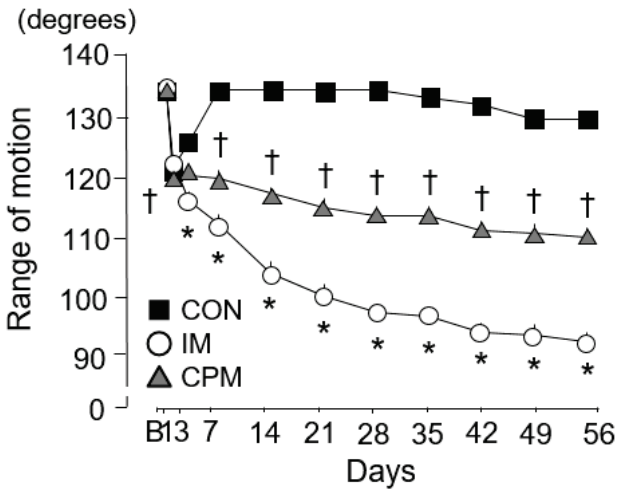


**Fig. 5.** Time course changes in the range of motion (ROM) of the knee joints on flexion. Data are presented as the mean ± SE. * $p<0.05$ continuous passive motion (CPM) versus the control (CON) group. † $p<0.05$ immobilization (IM) versus CPM group. B, baseline

In the present study, the results of knee joint transverse diameter and PPT at 1 day after injection indicated that injection of carrageenan produced acute inflammation and primary hyperalgesia in the affected joint. Moreover, these results indicated that injection produced the same level of arthritis in all animals.

The PPT in the control group increased to a level higher than the baseline after day 21. Previous study reported that the increase of the PPT in rats was associated with the alteration of their body weight gain (Luis-Delgado *et al*. 2006). In this study, the rats of the control group gained their weight gradually with aging (date are not shown). Therefore, the PPT in the control group increased with the growth of the rat. The PPT in the IM group was significantly decreased compared to the control group at 14 days after injection, which indicates that immobilization after induction of inflammation prolongs the recovery of mechanical hyperalgesia. In contrast, a significant increase in the PPT was confirmed in the CPM group compared to the IM group. Additionally, there were no significant differences between the CPM and control groups. Our findings revealed that CPM initiated after the onset of arthritis promotes the recovery of inflammatory-induced primary hyperalgesia. The CPM performed immediately after the induction of knee joint inflammation inhibited the expression of inflammatory cytokine and induced the expression of the anti-inflammatory cytokine (Ferretti *et al*. 2005). In this study, CPM of inflamed joints may have evoked a beneficial biological reaction that promoted the recovery of primary mechanical hyperalgesia. A significant decrease in the PPT was sustained in the IM group until day 56. Beginning 28 days after injection, the PPT in the CPM group was significantly decreased compared to the control group. In addition, there were no significant differences in the PPT between the IM and CPM groups from 42 to 56 days after injection. The knee joint immobilization for 6 weeks enhanced the medial articular nerve activity in rabbits during rest and knee joint motion to levels similar to those found in inflamed knee joints (Okamoto *et al*. 1999). Immobilization of the rat forelimb for 4 weeks produced mechanical allodynia that was related to plastic changes in the dorsal horn neuron (Ushida and Willis 2001). Our results showed that long-lasting immobilization negated the beneficial effects of CPM and produced hyperalgesia derived from plastic changes in the neurons.

Although the CGRP expression intensity in the deep layer (laminae III-VI) of L2-3 was significantly higher in the IM and CPM groups compared to the control group, there were no significant differences between these two treatment groups. It is known that CGRP increases the discharge frequency of WDR neurons in the dorsal horn, which is blocked by the CGRP receptor antagonist CGRP8-37 (Yan and Yu 2004). This indicates that increased CGRP expression in the spinal dorsal horn reduces the pain threshold through activation of WDR neurons, which are distributed in the deep layer of the spinal dorsal horn. Therefore, we considered that the decrease in PPT in the IM and CPM groups, at least after 56 days, was induced by central sensitization.

In this study, sensitivity of the hind paw was tested with 4 g von Frey filaments for mechanical allodynia and 15 g filaments for mechanical hyperalgesia. Allodynia is defined as a pain due to a stimulus that does not normally provoke pain, and hyperalgesia is an increased response to a stimulus which is normally painful. The increased PWR at 1 day after injection in all groups demonstrated mechanical allodynia and hyperalgesia in locations distal from an inflamed joint. Injection of carrageenan into deep tissues activates the dorsal horn neurons causing central sensitization (Neugebauer and Schaible 1990). Central sensitization is usually observed in the areas adjacent to the injury and

sometimes in distal locations (Radhakrishnan *et al.* 2003), manifested as secondary hyperalgesia (Sluka and Westlund 1993). In the IM group, mechanical allodynia and hyperalgesia in the hind paw were sustained until 56 days after injection. In contrast, in the CPM group, mechanical allodynia in the hind paw was not seen from 49 to 56 days. Moreover, mechanical hyperalgesia was not seen until 35 days after injection. This may indicate that CPM reduces mechanical allodynia and hyperalgesia in arthritis and may inhibit central sensitization in the spinal dorsal horn. However, after 42 days injection, secondary hyperalgesia in the CPM group was confirmed, which was mild compared to that of the IM group. Previous study demonstrated that eight-week joint immobilization induced hyperalgesia to mechanical stimulation associated with central sensitization in the spinal cord (Hamaue *et al*. 2013). This may influence the decrease in the pain threshold in the CPM group. Therefore, secondary hyperalgesia in the IM and CPM groups is caused by inflammation or immobilization.

In the superficial layer (laminae I-II) of the spinal dorsal horn in L4-5, CGRP expression intensity was significantly higher in the IM group compared to the control group, whereas no significant differences were seen between the control and CPM groups. Although CGRP expression intensity in the deep layer (laminae III-VI) of the spinal dorsal horn was significantly increased in the IM and CPM groups compared to the control group, there were no significant differences between the treatment groups. CGRP released into the superficial layer (laminae I-II) of the spinal dorsal horn induces hypersensitivity *via* the increased release of substance P and other neuropeptides (Kangrga and Randic 1990, Sun *et al.* 2004). Our results indicate that CPM inhibits the central sensitization induced by immobilization during the acute phase of arthritis. This is the one of the reasons why mechanical hyperalgesia in the CPM group was mild compared to the IM group. It was found that the mobilization of the inflamed knee joints of rats at 4 weeks increased the mechanical withdrawal threshold (Sluka *et al.* 2006). The authors discussed the possibility of the involvement of non-opioid pathways in the descending inhibition using serotonin and noradrenaline to produce analgesia. In the current study, descending inhibition may affect the decrease in secondary hyperalgesia.

One day after injection, the ROM was significantly decreased compared to those at baseline in all groups, with significantly increased ipsilateral knee joint transverse diameter, which indicated knee joint swelling by acute inflammation. Increases in the synovial fluid induced an increase in the intra-articular pressure and a decrease in the joint angle (Wood *et al.* 1988). Therefore, the limitations in ROM on 1 day after injection can be attributed to the development of swelling of the knee joint. The ROM in the IM and CPM groups was significantly decreased from 3 to 56 days after injection compared to the control group. The time-dependent limitation in ROM in the IM and CPM groups was induced by cast immobilization, which is derived from myogenic (Honda *et al.* 2015) and arthrogenic changes (Akeson *et al*. 1973). The ROM in the CPM group was significantly increased compared to the IM group. These results suggest that CPM is useful for the management, not only of hyperalgesia, but also of ROM limitations.

In summary, CPM initiated after the onset of arthritis promotes the recovery of inflammatory primary hyperalgesia and prevents the development of secondary hyperalgesia by decreasing CGRP expression in the superficial layer of the spinal dorsal horn. Additionally, CPM can inhibit progression of the immobilization-induced joint contracture. Therefore, we believe that CPM initiated after the onset of arthritis is beneficial for acute and chronic pain management. However, this study has some limitations. First, this research evaluated the knee joint transverse diameter and pressure pain threshold according to the severity of arthritis. In order to clarify the effects of CPM on inflammation, alteration of inflammatory cytokines and histological changes in the synovium must be quantified from the acute phase. Second, we only examined the expression of CGRP in the spinal dorsal horn. However, there are many factors involved in the central sensitization such as activation of the glial cell and expression of other neurotransmitters, substance P, nitric oxide, and glutamate. Additionally, the analysis of CGRP was performed with only experimental endpoints. In order to elucidate the mechanism of central sensitization and biological mechanism of CPM for decreased primary and secondary hyperalgesia, further research is necessary.

## Conflict of Interest

There is no conflict of interest.

## Acknowledgements

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