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Analgesic effects of a 5-HT3 receptor antagonist in an animal model of complex regional pain syndrome

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Abstract. – OBJECTIVE: Complex regional pain syndrome (CRPS) is caused by injuries from fracture after trauma and orthopaedic surgical procedures in the hind limbs. The symptoms of CRPS include warmth, pain, allodynia, and hyperalgesia. It is known that 5-hydroxytryptamine 3 (5-HT3) receptors contribute to hyperalgesia, but their role has not yet been fully elucidated. This study investigated the mechanism of pain relief when a 5-HT3 receptor antagonist was administered in a CRPS animal model.

MATERIALS AND METHODS: To establish a CRPS animal model, 10-week-old Sprague-Dawley rats were used in the experiment. On the fourth week post tibial fracture surgery, we performed the von Frey test to measure mechanical allodynia. After performing behavioural tests, we collected blood and tissue samples after sacrificing the animals. Enzyme-linked immunosorbent assay and western blot were also performed.

RESULTS: The experimental tibia fracture model-induced CRPS animals elicited increased 5-HT3 receptor expression, and the 5-HT transporter was decreased in the brain stem after 4 weeks of surgical intervention. Additionally, in CRPS-induced animals, both the concentration of substance P and the level of interleukin 6 were increased peripherally and centrally. Treatment with the 5-HT3 receptor antagonist, ramosetron, exerted an analgesic effect in the paw withdrawal test and was dependent on the attenuation of the 5-HT3 receptor population with inflammatory pain mediators.

CONCLUSIONS: These data suggest that treatment with the 5-HT3 receptor antagonist, ramosetron, in experimental CRPS animal models alleviated pain-related behaviours and may be a new therapeutic option or potential therapeutic agent for patients with CRPS.

Key Words:

Complex regional pain syndrome, Tibial fracture, Pain, Ramosetron, 5-HT3 receptor, Brainstem, 5-HT transporter, Locomotor activity, Allodynia, von Frey test.

Abbreviations

CRPS: complex regional pain syndrome; IL: interleukin; TNF: tumour necrosis factor; 5-HT: 5-hydroxytryptamine; CNS: central nervous system; 5-HT3R: 5-HT3 receptor; CTR group: sham group; TFM group: tibia fracture model group; TFM + Ramo group: TFM treated with ramosetron 0.1 mg/kg group; PVDF: polyvinylidene difluoride; TBST: Tween 20-TBS; ANOVA: analysis of variance; S.D.: standard deviation.

Introduction

Complex regional pain syndrome (CRPS) is a form of nociplastic pain that results from traumatic insults and has a prevalence of approximately 5.4–26.2 per 100,000-person years[1,2]. CRPS often develops due to injuries, including fractures and traumatic tissue injury, and is frequently induced by distal tibia and radial fractures[3,4]. The symptoms of CRPS include distal limb oedema, warmth, pain, allodynia, and hyperalgesia. Also, patients with CRPS can experience very poor quality of life due to pain, as well as sleep, anxiety and psychological problems[5,6]. The exact cause of CRPS is still unclear, and many studies have reported that neuropeptides, such as substance P contribute to the symptoms of patients with CRPS[7,8]. Substance P is synthesised in the C-fibre of sensory neurons and functions as a neuropeptide that promotes pain recognition. Substance P is also known to induce the synthesis of inflammatory cytokines, such as interleukin 1 (IL-1) and IL-6 in astrocytes and microglia[9]. It is also known to increase tumour necrosis factor α (TNF-α) and IL-6 levels in the fluid contained in blisters that are found in patients with early CRPS[10]. Similarly, in CRPS animal models, the increase in

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substance P and CRPS, which are the mediators of spinal inflammation, and TNF, IL-1β, and IL-6 are increased[11]. Therefore, many studies have reported the expression of substance P and inflammatory cytokines in CRPS models.

Furthermore, 5-hydroxytryptamine (5-HT; serotonin) is considered to play an important role in pain control in the central nervous system (CNS) and in the peripheral nervous system[12]. The descending pathway of 5-HT in the CNS has an inhibitory or facilitating effect on spinal processing of nociceptive information, depending on the acute or chronic pain condition and the type of receptor that is acted upon[13-15]. Among the various types of receptors for 5-HT, the 5-HT3 receptor (5-HT3R) is a non-selective ligand-gated ion channel. Although 5-HT3R is in the central and peripheral parts of the nervous system, its role is not fully understood; however, 5-HT3R is known to contribute to descending facilitatory mechanisms and hyperalgesia. In contrast, treatment with a 5-HT3R agonist, such as m-chlorophenylbiguanide, increased the excitability of C-fibre axons in sural nerves of rats[16]. A clinical study reported that administration of the 5-HT3R antagonist, granisetron, abolished the hyperalgesia induced by injection of 5-HT in the masseter muscle of the jaws of healthy individuals[17]. Ondansetron, a 5-HT3R antagonist, also significantly reduces mechanical allodynia and relieves pain in neuropathic pain[18,19]. Hence, 5-HT3R antagonists are known to have antinociceptive effect[20-23]. However, the nociceptive effects of ramosetron with regard to pain have not been studied. In this study, we investigated the effect of ramosetron on a CRPS animal model induced by tibial fractures.

Materials and Methods

Animals and Ethical Consideration

We used 10-week-old Sprague-Dawley rats (Osan, Korea). The rats were acclimatised to controlled laboratory conditions (temperature, 21 ± 2°C; humidity, 40%-60%, 12-hr light/dark cycle) for one week prior to experimentation to minimise stress levels. Food and tap water were provided ad libitum. The subjects were categorised into one of three groups: the sham (CTR) group, the tibia fracture model (TFM) group, or the TFM treated with ramosetron 0.1 mg/kg (TFM + Ramo) group. All procedures and research protocols in this study were approved by the Experimental Animal Ethics Committee at Wonkwang University (permit number WKU15-89).

Tibial Fracture Surgery

We performed tibial fracture operation under sevoflurane anaesthesia with nitrous oxide. After exposing the distal tibia, the midpoint of the right distal tibia was fractured using a microdrill and then closed with sutures (Figure 1). The tibia fractured subjects were unilaterally wrapped with cast tapes from the ankle to the hip of the fractured hind limb and returned to their home cage. The cast material was re-wrapped in a wire mesh to prevent the animals from chewing at their casts. Four weeks postoperatively, the casts were removed after the subjects were anaesthetised with sevoflurane.

Drug

Rats in the TFM + Ramo group were administered ramosetron (0.1 mg/kg) for 7 consecutive days, 3 weeks post tibia fracture surgery. The CTR and TFM groups were administered saline by subcutaneous injection.

Von Frey Tests

Rats were placed in an acrylic box on wire netting on the floor for 15 min. The allodynia was calculated using the 50% threshold of the avoidance response to von Frey filaments[24,25].

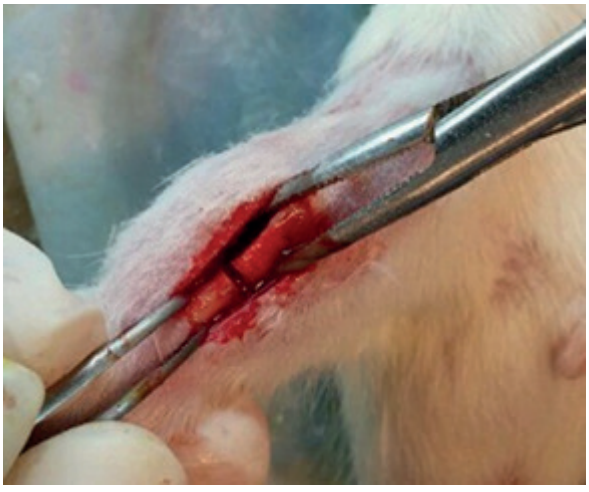

Figure 1. Representative photograph of a TFM operation. The midpoint of the right distal tibia was fractured using a microdrill.

$$50\%\ \text{g threshold} = \frac{(10\ txf + k\sim l)}{10{,}000}$$

where = value (in log units) of the final von Frey test used, k is the tabular value for the pattern of positive/negative responses, and ~ = mean difference (in log units) between stimuli (0.224).

Open-Field Test

The subjects were positioned at the centre of an acrylic rectangular lit arena for exploration, and their locomotive activities were tracked using an open-field apparatus (ENV-520, Med-Associates, VA, USA). Ambulatory activity was assessed using infrared beam interruption counts as measures for 30 min of exploration.

Plasma Concentration of Substance P

After euthanasia, blood samples were collected in heparinised tubes and then placed on ice. Aprotinin was added within 5 min of collection. The samples were centrifuged for 15 min at 1,000 × g within 30 min of collection. We followed the assay procedure described in the R&D kit (SKGE007; SKGE007; R&D Systems, Minneapolis, Minnesota, United States).

Protein Expression Analysis

Following decapitation of the rats and brain extraction, the brain tissue was microdissected into distinct parts on a cold glass plate and snap frozen in liquid nitrogen. Tissue samples were homogenised in a buffer solution (25 mM Tris, 1 mM ethylene glycol tetraacetic acid, 1 mM dithiothreitol, 0.1% Triton-X 100, pH 7.4) for protein extraction and stored on ice for 1 h. Tissue lysates were subsequently centrifuged at 12,000 × g at 4° C, and the supernatant was isolated and quantified using the BCA protein assay (Thermo, Rockford, USA) and a spectrophotometer (Molecular Devices, CA, USA). After the protein quantification assay, tissue lysates were diluted in 5X sample buffer (Elpis Biotech Inc., Korea) and shaken at 95°C for 5 min. The protein samples were then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Electrophoresis was performed at 80 V for 2 h, and protein samples were subsequently transferred to a 0.45 µm polyvinylidene difluoride (PVDF; Millipore Co., USA) membrane. To prevent non-specific binding of primary antibodies to the PVDF membrane, protein samples were incubated in 5% skim milk for 1 h at room temperature and washed in 0.05% Tween 20-TBS (TBST) for 10 min, thrice. The primary antibodies were α-5-HT transporter (1:1,000), α-5-HT3 receptor (1:1,000), α-IL-6 (1:1000), IL-1β (1:1,000), and α-glyceraldehyde 3-phosphate dehydrogenase (1:1,000). Protein samples were incubated using primary antibodies overnight at 4°C and washed twice with TBST for 10 min. Samples were then incubated with the secondary antibody, α-rabbit IgG (1:5,000), for 1 h at room temperature and washed in TBST four times. Detection was performed using a chemiluminescence analyser (Vilber Lourmat, France) with ECL reagent (Millipore Co., CA, USA).

Statistical Analysis

Statistical data and graphs were generated via analysis of variance (ANOVA) using Prism 4.0 (GraphPad software, CA, USA). The results of the behavioural experiment were expressed as mean ± standard error of the mean, and all other values are expressed as mean ± standard deviation (S.D). Data were determined to be statistically significant if the *p*-value was less than 0.05. A post-hoc analysis was conducted on the statistically significant data.

Results

Ramosetron Attenuated Nociceptive Behaviour Induced by Tibia Fracture

The von Frey test was performed to assess whether the nociceptive behaviour induced by tibial fracture could be reduced through treatment with ramosetron. Mechanical allodynia was measured at 4 weeks post-tibia fracture in rats. The 50% threshold (g) value of the sham (CTR) group was observed to be 15 ± 0, and that of the TFM group was measured to be 3.67 ± 0.68 at 4 weeks after tibial fracture surgery. The TFM + Ramo group (8.072 ± 2.063) showed a decrease in mechanical allodynia (Figure 2A).

The open-field test was used to determine whether the explorative behaviour induced by tibia fracture and ramosetron influenced the anxious pattern of behaviour (Figure 2B). Compared to the CTR group (19,280 ± 1,819), the TFM group (28,762 ± 2,045) showed a significantly higher increase in ambulatory activity, but the TFM + Ramo group (26,066 ± 1,008) showed no significant increase. There was no significant decrease between the TFM and TFM + Ramo groups.

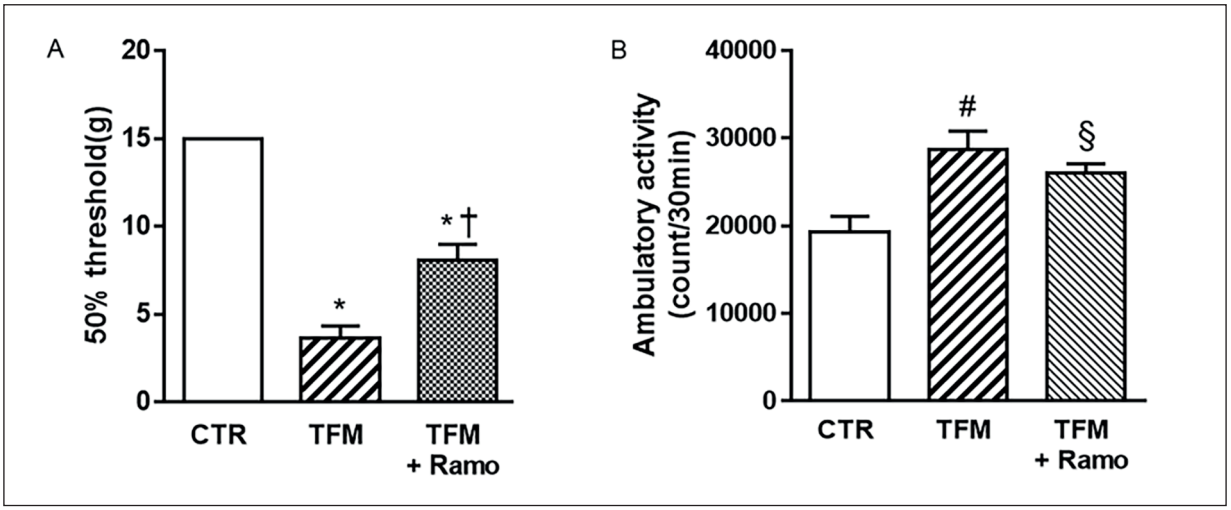

Figure 2. Paw withdrawal test for mechanical allodynia by von Frey test (**A**) and the ambulatory activity for exploration of the open-field apparatus in the TFM + Ramo group (**B**). Ambulatory activity was measured for 30 min and subjects were exposed to a novel environment for the first time. (**A**) Data represent mean ± S.E.M *$p < 0.001$ *vs.* the CTR group, †$p < 0.001$ *vs.* the TFM group by ANOVA (post-hoc with Fisher's PLSD). (**B**) #$p = 0.003$ *vs.* CTR group, §$p = 0.0404$ *vs.* CTR group by ANOVA (post-hoc with Fisher's PLSD).

Ramosetron Reduced 5-HT3 Receptors in Tibia Fracture Models

To ascertain the change in 5-HT3R following tibia fracture and 5-HT3R inhibition by ramosetron in the brain stem, the expression of 5-HT3R was assayed using western blot analysis. In addition, the expression of 5-HTT was confirmed. As shown in Figure 3, the expression of 5-HT3R was increased in the TFM group, and this was decreased by the administration of ramosetron for 7 consecutive days. Also, the expression of 5-HTT was significantly decreased in the TMF model compared to the CTR group.

Ramosetron Reduced the Increase in Substance P and IL-6 by Tibial Fractures

The above results confirmed that nociceptive behaviour following tibia fracture was alleviated by the administration of ramosetron. To observe the changes in the mediators related to inflammation after tibial fracture, we measured the levels of substance P in blood using an enzyme-linked immunosorbent assay (Figure 4). The level of substance P in blood was significantly higher in the TFM group (940.807 ± 55.92) than in the control group (696.091 ± 162.1) ($p < 0.05$). The level of substance P in the TFM + Ramo (728.627

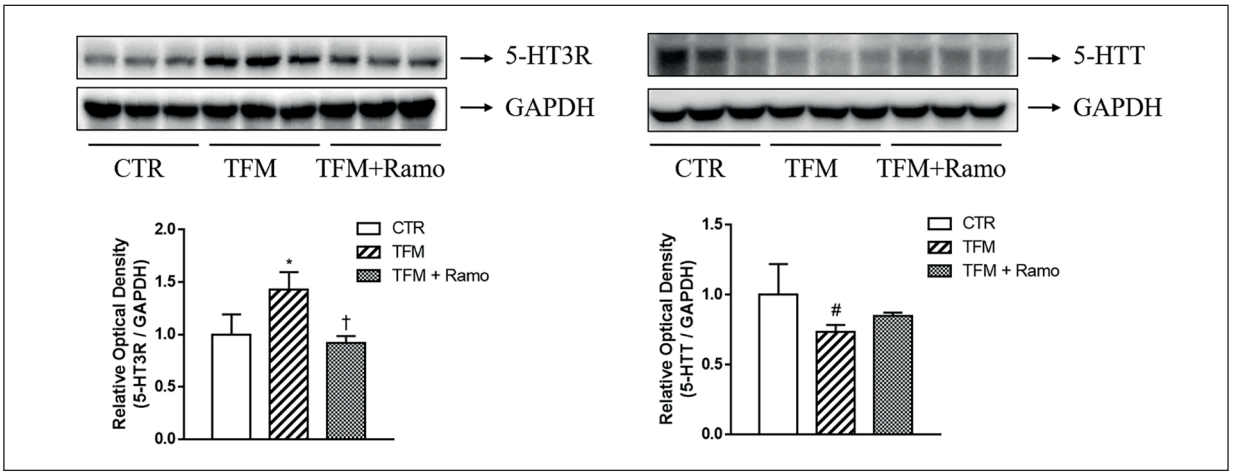

Figure 3. Representative western blot for 5-HT3R, 5-HTT in the brainstem of the TFM + Ramo group. Data represent mean ± S.D. *$p = 0.012$ *vs.* CTR group, †$p = 0.005$ *vs.* the TFM group, #$p = 0.016$ *vs.* CTR group by ANOVA (post-hoc with Fisher's PLSD).

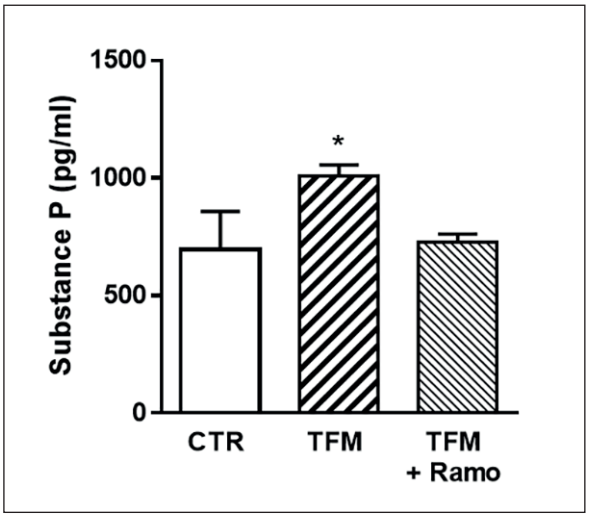

Figure 4. Concentration of substance P in the blood of TFM + Ramo group. Data represent mean ± S.D. *$p = 0.035$ *vs.* CTR group by ANOVA (post-hoc with Fisher's PLSD).

± 19.19) group was similar to that in the control group. It was confirmed that ramosetron administration reduced the level of substance P in the TFM group.

The expression of the cytokines TNF-α, IL-6, and IL-1β was analysed. There was no difference between the groups in terms of the expression of TNF-α and IL-1 β in the brainstem. However, the level of IL-6 expression was increased by about 1.8 times in the TFM group compared to the levels in the CTR group, and its level in the TFM + Ramo group was significantly decreased.

Discussion

This research was conducted to determine the effect of a 5-HT3 receptor (5-HT3R) antagonist on the pain threshold for mechanical stimulation in tibia fracture rat models. The subjects were examined using the von Frey test for mechanical allodynia 4 weeks after unilateral tibial fracture surgery for CRPS modelling.

It is well known that ramosetron is a 5-HT3R antagonist and it is mainly used as an antiemetic drug to suppress nausea and vomiting caused by chemotherapy. According to several studies, the administration of 5-HT3R antagonists was effective in CRPS clinical trials and laboratory animal studies. In this study, ramosetron was administered to the experimental CRPS model to determine its pain suppression or analgesic properties.

The results showed that chronic inhibition of 5-HT3R can cause antinociceptive effects in TFMs. Mechanical allodynia induced by tibia fracture was alleviated by ramosetron administration for 7 consecutive days. The paw withdrawal test using von Frey filaments revealed that the 50% stimulation threshold level for mechanical allodynia was significantly lower in the TFM group than in the CTR group that had undergone sham operation (Figure 2A). Therefore, the tibial fracture model established surgically in this study is an experimental model of CRPS.

When first introduced into a novel environment, rodents exhibit exploratory behaviour as an innate behavioural pattern. CRPS animals in the TFM group showed a significant increase in ambulation count compared to those in the CTR group, but the TFM + Ramo group showed results that were similar to those of the CTR group (Figure 2B). The increase in ambulatory activity observed in the exploratory behaviour of the TFM group seems to be due to psychological instability, such as anxiety or fear, rather than pain. However, research on emotional and/or psychological instability caused by hyperalgesia has not been conducted; hence, further studies are required.

The levels of 5-HT3R and the expression of 5-HTT were confirmed in brainstem using western blot analysis, and 5-HT3R expression was inhibited by ramosetron administration. The density of 5-HT3R in the brainstem was increased in the TFM group and was significantly inhibited in the TFM + Ramo group (Figure 3A). The findings of a previous pain relief study on a different class of 5-HT3R antagonist, tropisetron, in terms of mechanical allodynia, strongly support our finding[26]. Compared to the change in 5-HT3R, the change in 5-HTT levels followed the inverse pattern, and the degree of decrease of 5-HTT in the brain stem was alleviated by the administration of ramosetron (Figure 3B). This is credible as the reduction of 5-HTT compared to that in other studies, has been reported to increase the risk of pain sensitivity[27-29].

In patients with CRPS, substance P is known to affect nociceptive transmission and the synthesis of inflammatory cytokines[7,8]. An increase in the levels of substance P in the blood was observed in the TFM group, and it was confirmed that it was reduced in the TFM + Ramo group (Figure 4). Although not studied in the CRPS or TFM groups, other disease as rheumatism studies have indicated that 5-HT3R antagonist effects reduce IL-6 levels[30,31].

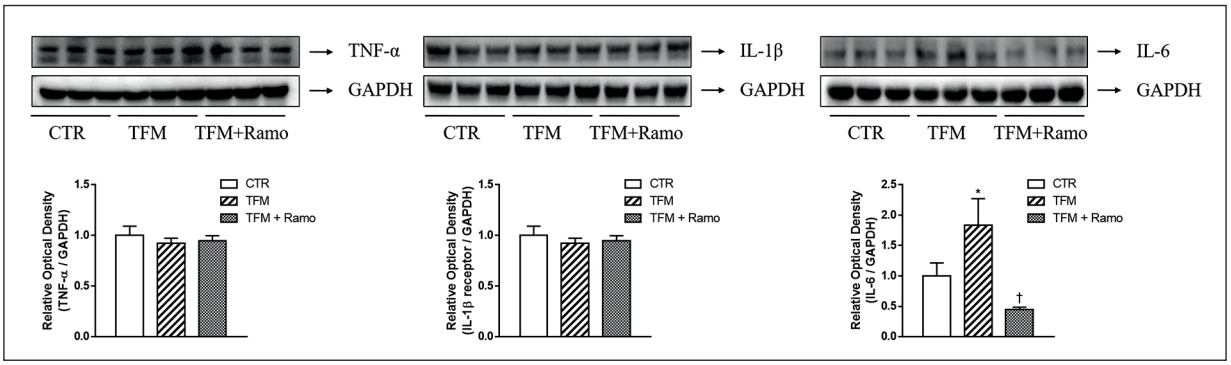

Figure 5. Representative western blot images and quantification of related optical densities for TNF-α, IL-1β and IL-6 in the brainstem of TFM + Ramo group. Data represent mean ± S.D. *$p = 0.011$ *vs.* CTR group, †$p = 0.001$ *vs.* the TFM group by ANOVA (post-hoc with Fisher's PLSD).

We confirmed that the expression of IL-6 was significantly increased in the brainstem of rats in the TFM group. However, the administration of ramosetron significantly reduced the expression of IL-6 in the TFM + Ramo group (Figure 5C). Administration of IL-6 induced mechanical allodynia or thermal hyperalgesia, while anti-IL-6 antibodies ameliorated pain-related behaviours[32,33]. Previous studies have reported that IL-6 expression is highly correlated with pain behaviour. Therefore, IL-6 receptor inhibitors attenuate mechanical allodynia and thermal hyperalgesia and have shown promise as a therapeutic tool for pain management in many studies[34]. When chronic contraction was applied to IL-6 knockout mice, hypersensitivity to skin heat and pressure was not clear, and this could be considered to play an important role in IL-6 hypersensitivity[35]. However, although the expression of IL-6 in the brainstem was significantly reduced by the administration of ramosetron in the TFM-induced CRPS model, expression in all brain regions was not investigated in this study. Unfortunately, IL-1β and TNF-α levels were not significantly altered in the brainstem.

Conclusions

This study provides evidence that shows the effects of the 5-HT3R antagonist, ramosetron, on the modulation of pain-related behaviours. Ramosetron treatment reduced mechanical allodynia response and the level of substance P in the blood of rats in the TFM group. In addition, ramosetron significantly reduced the expression of IL-6 in the TFM group. These data suggest that ramosetron therapy may be a new treatment option or potential drug therapy for CRPS.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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