



The Role of NLRP1 Inflammasome and Interleukin 1 β in Experimental Neuropathic Pain Model in Rat and the Effect of Tramadol Treatment

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ABSTRACT

AIM: To evaluate the effects of tramadol on inflammation by measuring NLRP1 and IL-1 beta (IL-1 β) levels in an experimental neuropathic pain model.

MATERIAL and METHODS: Sprague–Dawley rats were divided into three groups: control, chronic constriction injury (CCI), and CCI + tramadol. Neuropathic pain was assessed using mechanical allodynia, thermal hyperalgesia, and cold allodynia. IL-1 β and NLRP1 levels were evaluated using ELISA on sciatic nerve (SN), dorsal root ganglion (DRG), and serum either on day 3 or days 8 postsurgery.

RESULTS: On day 3, paw withdrawal latency (PWL) was lower in the CCI and CCI + tramadol groups than the control group in both mechanical and cold allodynia tests. On day 8, the PWL in the CCI group was also lower than in the control group. In contrast, tramadol increased the PWL on day 8 compared to day 3 in the CCI group. During cold allodynia, PWL decreased in the CCI group, however, tramadol reversed this effect on days 3 and 8. Tramadol, therefore, ameliorated pain hypersensitivity in mechanical/cold allodynia tests. Serum IL-1 β levels were higher in the CCI + tramadol and CCI groups than the control group, although serum IL-1 β levels in the CCI and CCI + tramadol groups were comparable. Tramadol decreased the IL-1 β and NLRP1 in DRG compared with the CCI group. A similar trend was observed in the SN samples.

CONCLUSION: Our experiments revealed an increase in IL-1 β and NLRP-1 levels in a neuropathic pain model and found that tramadol had an anti-inflammatory effect on the IL-1 β and NLRP1 inflammasomes.

KEYWORDS: Chronic constriction injury, IL1 β , Inflammasomes, Neuropathic pain, NLRP1, Tramadol

INTRODUCTION

Neuropathic pain is defined as “pain resulting from a disease or lesion affecting the somatosensory system,” negatively impacts quality of life (1). Neuropathic pain can originate from the peripheral and central nervous systems. Peripheral neuropathic pain includes diabetic neuropathic pain, carpal tunnel syndrome, posttraumatic neuropathy,

and postherpetic neuralgia, whereas central neuropathic pain includes poststroke pain, multiple sclerosis-related neuropathic pain, and pain due to spinal cord injury (7,22).

Studies have suggested that spinal pro-inflammatory cytokines, such as tumor necrosis factor (TNF) α , interleukin (IL)-6, and Interleukin-1 beta (IL-1 β), are responsible for the development of neuropathic pain via positive feedback communication

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between neurons and glia (14). Neuroinflammation after injury has also shown to alter the chemical microenvironment around sensory nerves, leading to peripheral sensitization. Neuroinflammation enhances neuronal membrane excitability and synaptic activity in the pain pathway (26).

IL-1 β is a well-known inflammatory mediator that activates the immune system by binding to IL-1 receptors. Previous studies have shown that IL-1 β , a pro-inflammatory cytokine that causes neuroinflammation, also plays a role in the pathogenesis of neuropathic pain (14,26).

Inflammasomes are nod-like receptors that induce the maturation of pro-IL-1 β to IL-1 β in the presence of stimuli (17,18). Inflammasomes activate procaspase-1 that activates caspase-1 via the inflammasome adaptor protein. Caspase-1 activation promotes maturation of pro-inflammatory cytokines, such as IL-1 β and IL-18. Therefore, inflammasome-activated caspase-1 activates IL-1 β and IL-18 (18). Nod-like receptor protein 1 (NLRP1) inflammasomes is found in the central nervous system (CNS) and spinal cord. The NLRP1 inflammasome is mainly found in neurons and comprises NLRP1, caspase-1, caspase-5, and apoptosis-associated speck-like protein containing CARD (ASC) (4). Studies have shown that high levels of the NLRP1 inflammasomes are present during brain and spinal cord injuries (5,6). Previous studies have revealed the effect of inflammasomes on IL-1 β activation and the effect of IL-1 β on neuropathic pain, but few studies have examined the effect of the NLRP1 inflammasome on neuropathic pain (12,14).

Tramadol, a weak opioid and serotonin–norepinephrine reuptake inhibitor, is used to alleviate neuropathic pain (25). The inhibitory effect of tramadol on IL-1 β has previously been demonstrated (20); however, its effect on the NLRP1 inflammasome is unknown.

This study aimed to conduct behavioral experiments (mechanical allodynia, thermal hyperalgesia, and cold allodynia) and evaluate the serum IL-1 β and NLRP1 levels in rats before and after inducing pain with chronic constriction injury (CCI) using ELISA. Additionally, determine the effect of tramadol in this experimental neuropathic pain model.

■ MATERIAL and METHODS

Animal handling and experimental procedures were performed in accordance with the National Laboratory Animal Use and Care Guidelines and approved by the Eskisehir Osmangazi University Animal Experimentation Local Ethics Committee (Date: 27.11.2020, Decision No.: 737-1).

Animals

We used 30 adult male Sprague–Dawley rats (n=30) weighing 160–220 g. The animals were kept in controlled environmental conditions under a temperature range of 22°C–23°C, humidity of 60 \pm 5%, 12-h light–dark cycle, and with free access to food and water ad libitum. Experiments began after a week of habituation, and all behavioral experiments were performed between 9 a.m. and 2 p.m.

Drugs

A single dose of paracetamol (25 mg/kg) was administered to the rats on the day of surgery. CCI was performed on all rats under ketamine–xylazine anesthesia (80 and 8 mg/kg, intraperitoneally [i.p.]). Surgery was limited to the right sciatic nerve (SN). Tramadol (10 mg/kg i. p.) was administered to the CCI + tramadol group 45 min before the operation and for 9 days after the operation.

Surgical Operation and Experimental Groups

The rats were divided into three groups (n=10): sham (control), CCI, and CCI + tramadol. In the sham group, an incision of approximately 1 cm was performed in the biceps femoris under aseptic conditions and anesthesia. After muscle separation, the incision was closed with 4.0 silk without visualizing and binding the SN. Based on the original procedure reported previously, the CCI model was used to induce neuropathic pain in rats (3). The right SN was exposed via a mid-thigh incision. The nerve was freed of connective tissues by roughly 7 mm proximal (1–2 mm) to the sciatic trifurcation point, and two ligatures (4–0 Ethicon Chromic Catgut) were tied loosely around it with a 1.0–1.5 mm distance between each. Finally, a 4.0 silk thread was used to seal the incision (24). The same researcher (E. Y.) performed all the surgeries. In the third group, tramadol administration was started 45 min before the operation and continued for 9 days after the operation.

Sample collection

The affected nerves (n=5) and associated dorsal root ganglions (DRG, n=5) were assessed for IL-1 β and NLRP1 levels before euthanasia on the third and eighth day. On the third day (acute period) and eighth day (chronic period) postsurgery in separate groups, ketamine–xylazine (80/8 mg/kg) anesthesia was administered, and blood was collected from the tail vein of the rats with a 25 gauge needle (20). The blood was centrifuged at 1,500 g for 10 min at 4°C. Serum samples were taken from the tail vein of the rats under anesthesia on days 0, 3, and 8 and stored at –80°C for ELISA. A 2-cm piece of the SN was taken, including the area of the ligatures. DRGs were extracted from both sides of the spinal cord. The tissues were weighed and placed separately in a 300.25- μ L medium containing 300 μ L of Cell Lytic-MT mammalian tissue lysis–extraction reagent (Sigma Chemical Co., USA) and 0.25 μ L of protease inhibitor cocktail (Sigma Chemical Co., USA) (10).

Behavioral Experiments

The rats were tested for mechanical allodynia, thermal hyperalgesia, and cold allodynia before the operation, day 0, and on the third day and eighth day after the procedure. All behavioral experiments were performed simultaneously each day by a single observer between 9 a.m. and 2 p.m.

Mechanical plantar test: Mechanical allodynia

Before the experiments, the animals were acclimated in six-wire mesh cabinets for 30 min. Mechanical allodynia (23) was assessed using an electronic dynamic plantar esthesiometer (Ugo Basile S.R.L. 37400-002, Italy). The pointed tip of the esthesiometer (diameter of 0.5 mm) was placed on the mid-

plantar surface of the animals' right hind paw with a vertically increasing force (0–50 g in 20 s). PWL was defined as the time elapsed between the tip of the dynamic plantar apparatus making contact with the paw and the hind paw retracting. Time was recorded using an automated sensor. The PWL was measured in seconds.

Thermal hyperalgesia

The thermal nociceptive threshold was measured by applying radiant heat to the mid-plantar surface of the right hind paw using a thermal plantar test device (Ugo Basile S.R.L. 37370-002, Italy) (23). The rats were placed in cabinets 30 min before the measurements to acclimatize to their environment. The PWL was automatically recorded. The cutoff value for probable tissue damage in the animals' paws was set at 20 s.

Cold plate test: Cold allodynia

A cold plate test (Hot/Cold Plate, Ugo Basile S.R.L., Italy) was performed to evaluate cold allodynia in the hind paws of the rats (1). Before the measurement, the rats were kept at under cold temperature ($5 \pm 0.5^\circ\text{C}$) for adaptation. After a 2-min adaptation period, the rats' claw pulling and holding times placed on the cold plate at $5 \pm 0.5^\circ\text{C}$ were observed for 3 min. The time they first lifted their paws was recorded as latency. The results are expressed as the mean second \pm standard error of the mean (SEM).

Evaluation of IL-1 β and NLRP1 levels using ELISA

IL-1 β and NLRP1 levels in serum, SNs, and DRG samples were determined using the rat ELISA kit in accordance with the kit procedure (YL Biont ELISA Kit, Shanghai YL Biotech Co., Ltd., Shanghai, China). On days 0, 3, and 8, blood samples were obtained from the rats' tail vein under anesthesia in collecting tubes containing ethylene diamine tetraacetic acid, then centrifuged at 3,000 rpm for 20 min on ice. Plasma samples were stored at -80°C until they were used. A 2-cm piece of the SN, including the area containing ligatures and associated DRGs, was isolated from five rats in each of the three groups on days 3 and 8 under ketamine–xylazine anesthesia. On these days, the homogenized SN and DRG tissues were lysed by adding 0.25 μL of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri) into 300 μL of Cell Lytic-MT tissue extraction solution (Sigma-Aldrich, St. Louis, Missouri). Tissues were centrifuged for 10 min at 12,500 g. The supernatants were stored at -80°C until the ELISA was performed. The absorbance was measured spectrophotometrically at 450 nm using a Biotek ELx800 ELISA reader (BioTek Instruments, Winooski, Vermont). NLRP1 levels are shown in ppg/mL (medium)/mg protein, and IL-1 β levels were shown as ng/L/mg protein (10).

Statistical Analysis

Data are expressed as mean \pm SEM. The significance between groups was determined using two-way repeated measures analysis of variance (ANOVA). Multiple comparisons between groups were performed using Tukey's test. All statistical analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, New York). The statistical significance was set at $p < 0.05$.

RESULTS

Behavioral Tests

Before the operation, behavioral experiments were performed in all three groups. Thermal hyperalgesia test results revealed insignificant differences (data not shown).

Mechanical allodynia

On day 0, there was insignificant difference among the three groups in the mechanical allodynia test. These measurements were considered baseline measurements, and these results showed homogeneity among the groups at the beginning of the experiment.

In the control group, there were insignificant differences between measurements on days 0, 3, and 8. The PWL of the CCI group was significantly lower on days 3 and 8 than the baseline value ($p < 0.001$). The PWL of the CCI + tramadol group was significantly lower on day 3 than at baseline ($p < 0.001$); however, increased on day 8 ($p = 0.001$). On day 3, PWL was significantly lower in the CCI and CCI + tramadol groups than in the control group ($p < 0.001$). However, on day 8, the CCI group showed a significant decrease compared to the control group ($p = 0.028$), whereas the CCI + tramadol group showed a significant increase compared to the CCI group ($p = 0.015$, Figure 1).

Cold allodynia

There were insignificant differences between the groups during the baseline measurements (day 0) of the cold allodynia test, confirming homogeneity of the groups. There were insignificant differences in the PWL of the animals on days 0, 3, and 8 in the control group. There was a significant decrease in PWL in the CCI group on days 3 and 8 ($p < 0.001$) compared to the baseline. Furthermore, PWL reduction in the CCI group was greater on day 8 than on day 3 ($p = 0.005$). Similarly, PWL in CCI + tramadol was significantly lower on day 3 and day 8 than the baseline ($p < 0.001$). In addition, in this group, the PWL decreased further on day 8 than day 3 ($p = 0.041$). When the day 3 measurements of the groups were compared, the PWL in the CCI and CCI + tramadol groups decreased significantly compared to the control group ($p < 0.001$ and $p = 0.003$, respectively). Day 8 PWL measurements in the CCI and CCI + tramadol groups, were significantly lower than in the control group ($p < 0.001$). Interestingly, the PWL on day 8 was significantly higher in the CCI + tramadol group than in the CCI group ($p < 0.001$, Figure 2).

IL-1 β level in serum

One of the aims of this study was to investigate the alterations of IL-1 β levels in serum, DRG, and SNs after exposure to the CCI experimental model of neuropathic pain using ELISA. CCI is used for the slow development of edema and slow progression of nerve damage. IL-1 β levels were measured (246.75 ± 18.05 ng/L) three days after the procedure, indicating a significant increase after comparing the baseline measurements of the CCI group (70.26 ± 2.69 ng/L) and sham group (73.85 ± 2.75 ng/L). This was a statistically significant difference ($p < 0.001$). Eight days after the procedure, there was a signif-

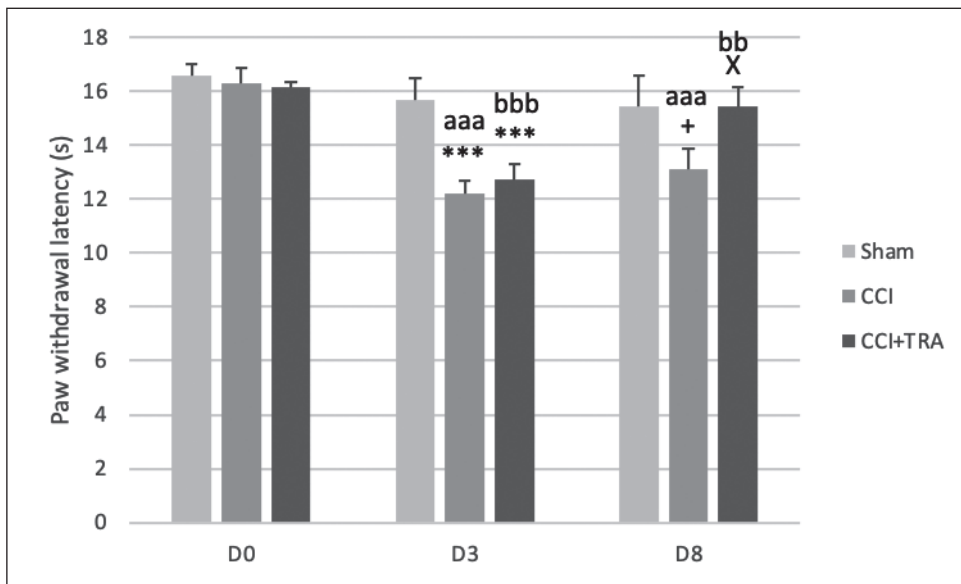


Figure 1: The paw withdrawal latency in mechanical allodynia test. ^{***}: p<0.001 vs control, +:p<0.05 CCI vs control on D8; x: p<0.05 CCI vs CCI+TRA on D8; aaa:p<0.001 D0 vs D3 CCI group; bb: p<0.01 D3 vs D8 CCI+TRA group, bbb: p<0.001 D0 vs D3 CCI+TRA group.

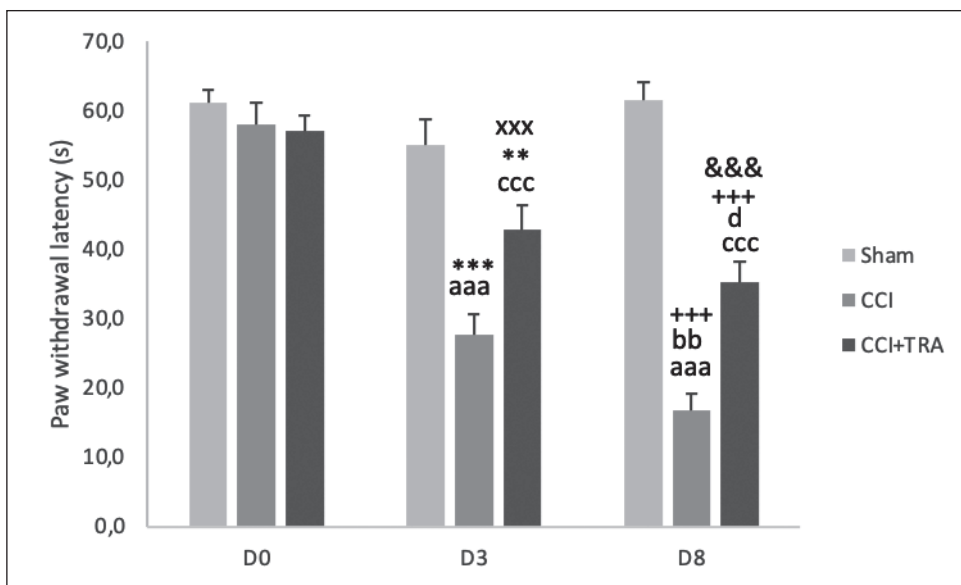


Figure 2: The paw withdrawal latency in cold allodynia test. ^{**}: p<0.01 Sham vs CCI+TRA in D3; ^{***}: p<0.001 Sham vs CCI in D3; ^{xxx}: p<0.001 CCI vs CCI+TRA in D3; ⁺⁺⁺: p<0.001 sham vs CCI and sham vs CCI in D8; ^{aaa}: p<0.001 D0 vs D8 and D0 vs D3 in CCI group; ^{bb}: p<0.01 D3 vs D8 in CCI group; ^{ccc}: p<0.001 D0 vs D8 and D0 vs D3 in CCI+TRA group; ^d: p<0.05 D3 vs D8 in CCI+TRA group; ^{&&&}: p<0.001 CCI vs CCI+TRA group in D8.

icant increase in the IL-1 β levels of the CCI group (238.74 \pm 20.87 ng/L) compared to the sham group (71.28 \pm 2.04 ng/L, p<0.001). However, there was insignificant difference between the CCI and CCI + tramadol groups on days 3 and 8 (Figure 3 shows IL-1 β levels in serum samples).

IL-1 β level in DRG samples

Three days after the procedure, IL-1 β levels of the DRG samples on the CCI-affected side showed slightly higher values in the CCI group (247.70 \pm 29.80) than the sham group (178.04 \pm 12.48, p=0.019). On day 3, IL-1 β levels in DRG samples from the CCI + tramadol group (186.17 \pm 40.54) were lower than in the CCI group (p=0.037). On day 8, the IL-1 β levels in the CCI (346.42 \pm 26.64) and CCI + tramadol (265 \pm 26.92) groups further increased. There was a statistically significant difference between the CCI and

sham groups (p<0.001). Similarly, the increase in IL-1 β levels was statistically different between the sham and the CCI + tramadol groups (p=0.004). However, tramadol administration caused a significant reduction in IL-1 β levels when compared with the CCI group (p=0.007; Figure 4 shows the IL-1 β levels in DRG samples).

IL-1 β level in SN samples

Three days after the procedure, IL-1 β levels in the SN samples from the CCI-affected side (263.30 \pm 32.28) indicated a borderline increase in the CCI group compared to the sham group (183.25 \pm 2.08, p=0.059). Eight days after the procedure, IL-1 β levels in the SN samples were significantly higher in the CCI and CCI + tramadol groups than in the sham group (CCI: p<0.001, CCI + tramadol: p=0.004). There was no statistically significant difference between the CCI and CCI + tramadol

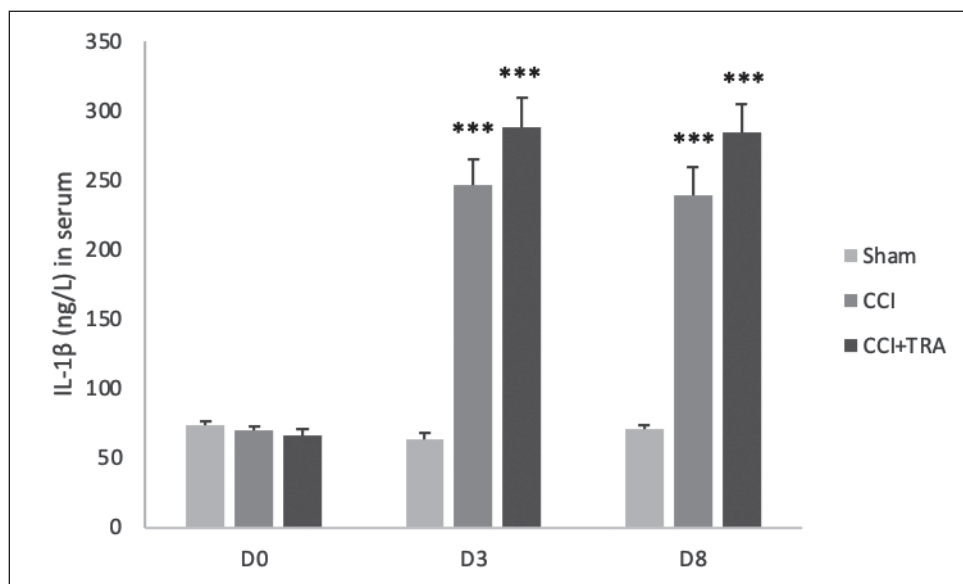


Figure 3: IL1 β level in serum samples before the operation (D0) and 3 and 8 days (D3 and D8) following surgery were quantified by ELISA. Data was expressed as mean \pm SEM of IL1 β levels. Statistically significant difference was shown as ***: $p < 0.001$ sham vs CCI within D3, sham vs CCI+TRA within D3; +++: $p < 0.001$ sham vs CCI within D8, sham vs CCI+TRA within D8.

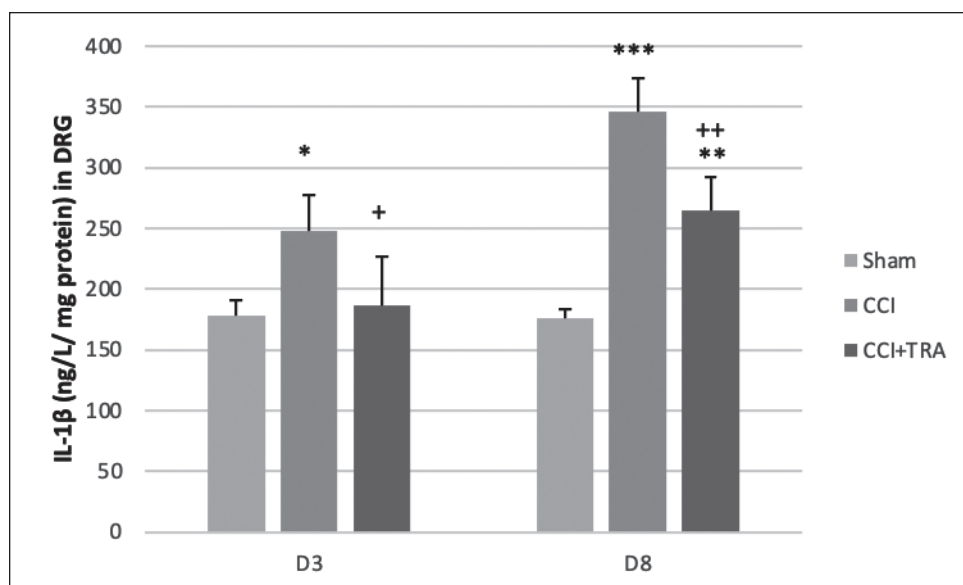


Figure 4: IL1 β level in DRG samples in 3rd and 8th days (D3 and D8) following surgery were quantified by ELISA. Data was expressed as mean \pm SEM of IL1 β levels. Statistically significant difference was shown as *: $p < 0.05$ sham vs CCI within D3; ***: $p < 0.001$ sham vs CCI within D8; **: $p < 0.01$ sham vs CCI+TRA within D8; +: $p < 0.05$ CCI vs CCI+TRA within D3; ++: $p < 0.01$ CCI vs CCI+TRA within D8.

groups. However, the trend of SN IL-1 β levels in the CCI + tramadol group was similar to that of IL-1 β levels in the DRG samples (Figure 5 shows IL-1 β levels in SN samples).

NLRP1 level in DRG and SN samples

In the study, NLRP1 levels in the serum samples on days 0, 3, and 8 could not be determined using ELISA. Therefore, the NLRP1 data in serum samples are not presented. On day 3, there was a significant increase in NLRP1 levels in DRG in the CCI group compared to sham group ($p = 0.017$). The CCI + tramadol group had a lower level of NLRP1 than the CCI group ($p = 0.051$). Eight days after the procedure, the CCI group showed a significant increase compared with the sham group ($p < 0.001$; Figure 6 shows NLRP1 levels in DRG samples).

The graphical illustration shows a pattern similar to NLRP1 levels in the DRG samples. However, there was no statistically significant difference in the NLRP1 levels in the SNs among the groups (Figure 7 shows the NLRP1 levels in SN samples).

DISCUSSION

IL-1 β , is a pro-inflammatory cytokine associated with many painful diseases (19,29). Pain can be caused by increased IL-1 β production during acute and chronic inflammation. IL-1 β stimulates the production of algogenic substances by directly affecting neuronal activity in the peripheral nervous system and CNS (21,27,28). Blocking the IL-1 β signaling pathway has proven effective in reducing hypersensitivity to inflammatory pain in several animal models (21). IL-1 β is acknowledged as a proinflammatory cytokine capable of heightening neuronal

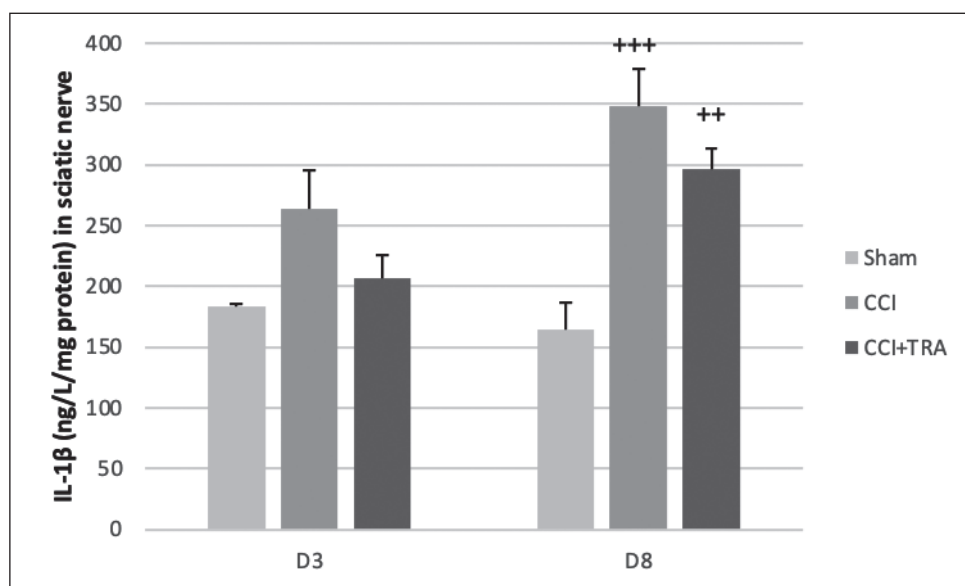


Figure 5: IL1 β level in sciatic nerve samples in 3rd and 8th days (D3 and D8) following surgery were quantified by ELISA. Data was expressed as mean \pm SEM of IL1 β levels. Statistically significant difference was shown as ++: $p < 0.01$ sham vs CCI+TRA within D8, +++: $p < 0.001$ sham vs CCI within D8

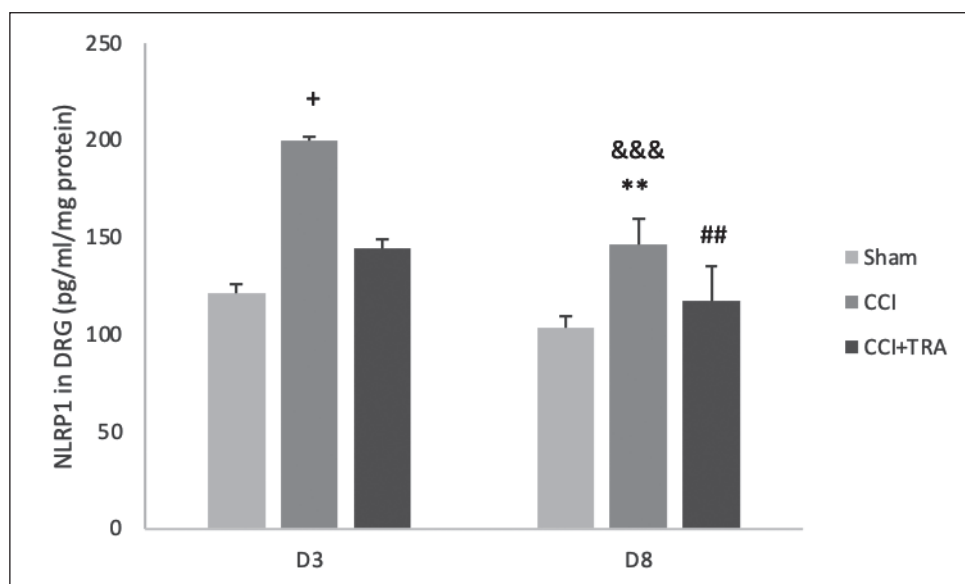


Figure 6: NLRP1 levels DRG samples in 3rd and 8th days (D3 and D8) following surgery were quantified by ELISA. Data was expressed as mean \pm SEM of IL1 β levels. Statistically significant difference was shown as +: $p < 0.05$ Sham vs CCI in D3; **: $p < 0.01$ D3 vs D8 in CCI group; &&&: $p < 0.001$ Sham vs CCI in D8; ##: $p < 0.01$ CCI vs CCI+TRA in D8.

sensitization (2,16), however, it also plays a role in modulating inhibitory neurotransmission (8,9). However, the mechanisms underlying the abnormal increase of IL-1 β levels are still being studied.

Inflammasomes (NLRP) are large intracellular multiprotein complexes that play a central role in innate immunity by activating caspases that converts pro-IL-1 β to active-IL-1 β (17,18). NLRP1 and NLRP3 are widely studied among all the inflammasomes (12,30). NLRP1 inflammasome, which is widely expressed in cells and tissues of the immune system and nonhematopoietic tissues, binds directly to ASC via the pyrin domain and caspase-1/caspase-5 via the CARD domain (17,18).

The relationship between inflammasomes and inflammatory cytokines has been explored in several studies that targeted

inflammasomes to regulate inflammation and reduce associated pain (13,15,26).

However, few studies have investigated the relationship between tramadol and IL-1 β levels in serum and peripheral tissues during acute and chronic neuropathic pain but the relationship between tramadol and NLRP1 levels has not been previously investigated (19). Miranda et al. suggested a synergistic effect of gabapentin and tramadol on IL-1 β and found that IL-1 β levels in the spinal cord were significantly lower in the tramadol, gabapentin, or combination group on the third and seventh days than in the streptozotocin group (19). Our findings support this study, given that we found a significant decrease in IL-1 β levels in DRGs in the CCI + tramadol group compared to the CCI group. A similar pattern was observed in the SN IL-1 β levels; however, this decrease

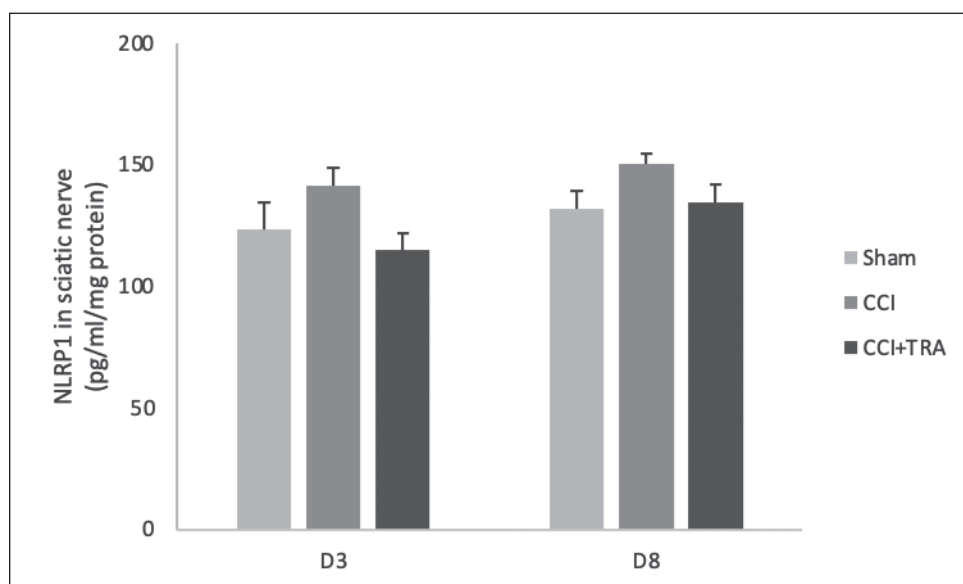


Figure 7: NLRP1 levels sciatic nerve (SN) samples in 3rd and 8th days (D3 and D8) following surgery were quantified by ELISA. Data was expressed as mean \pm SEM of IL1 β levels.

was statistically insignificant. Endogenous opioid peptides derived from immune cells that accumulate in areas of nerve damage are known to contribute to peripheral analgesia (11). Tramadol binds more to DRG opioid receptors due to endogenous opioid peptides released from inflammatory cells at the site of SN injury, which may have resulted in a strong anti-inflammatory effect in DRG. Since IL-1 β levels in the CNS were not evaluated in this study, further studies are needed to qualitatively determine IL-1 β levels in the CNS, specifically the astrocytes and spinal horn.

In the present study, the mechanical allodynia test revealed that neuropathic pain had developed in the CCI and CCI + tramadol groups on the third day. On the eighth day, the CCI + tramadol group had a stronger antinociceptive effect than the CCI group. Similarly, neuropathic pain occurred in the experimental groups on the third and eighth days of the cold allodynia test; however, a higher increase in latency was observed in the CCI + tramadol group than in the CCI group on both days.

Li et al. examined the effect of the inflammasome caspase-1 and IL-1 β on the CCI-induced neuropathic pain model and showed that NALP1/NLRP1 is activated in neuropathic pain in spinal astrocytes and neurons, particularly in the superficial lamina of the spinal cord dorsal horn. In the same study, rats with CCI were given repeated intrathecal injections of aspirin-triggered-15-epi-lipoxin A4 (ATL), which has the same potent effects as endogenous lipoxins, resulting in significant attenuation of CCI-induced thermal hyperalgesia (13).

Another study found that the NLRP1 inflammasome was activated in the hippocampus of rats exposed to CCI-induced chronic neuropathic pain (12).

In our study, we were unable to determine serum NLRP1 levels using ELISA. However, in the DRG measurements,

a statistically significant increase was observed in the CCI group compared to the control group on the third and eighth days, and a similar pattern, although without significance, was observed in the SN. The measurement on the eighth day in the DRG revealed a significant decrease in NLRP1 levels in the CCI + tramadol group compared to the CCI group. These results showed that the anti-inflammatory effect of tramadol in the inflammation cascade begins on the NLRP1 inflammasome before IL-1 β .

The main limitations of this study are the small number of rats used and the lack of advanced techniques, such as immunohistochemistry, and molecular tools, such as polymerase chain reaction for NLRP1 measurement.

CONCLUSION

The results obtained in the study showed an increase in IL-1 β and NLRP1 inflammasome levels after neuropathic pain, suggesting that the NLRP1 inflammasome is involved in the neuropathic pain pathway. The results also highlight that the role of tramadol in ameliorating neuropathic pain is supported by its anti-inflammatory effect, serotonin-norepinephrine re-uptake inhibitory effect, and opioid analgesic properties. Finally, the results shown that the anti-inflammatory effect of tramadol affects IL-1 β and the pro-inflammatory cytokine NLRP1 inflammasome, particularly in the DRG. Therefore, further studies are needed to strengthen the effect of IL-1 β and NLRP1 in the pathogenesis of neuropathic pain and their potential relationship with tramadol.

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AUTHORSHIP CONTRIBUTION

Study conception and design: TT, AB

Data collection: CCU, NH

Analysis and interpretation of results: CCU, TT

Draft manuscript preparation: SG, AB, EY

Critical revision of the article: SG, AB, EY

Other (study supervision, fundings, materials, etc...): CCU, NH, TT

All authors (TT, CCU, NH, EY, AB, SG) reviewed the results and approved the final version of the manuscript.

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