

Antiallodynic and antihyperalgesic activities of zerumbone via the suppression of IL-1 β , IL-6, and TNF- α in a mouse model of neuropathic pain

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Background: Neuropathic pain is a debilitating condition that severely affects the quality of life for those with this pain condition, and treatment for pain relief is greatly sought-after. Zerumbone (Zer), a sesquiterpene compound isolated from the rhizomes of a Southeast Asian ginger plant, *Zingiber zerumbet* (L.) Roscoe ex Smith. (Zingiberaceae), showed antinociceptive and antiinflammatory properties when previously tested on models of nociception and inflammation.

Objective: This study investigated the effects of prophylactic administration of zerumbone on allodynia and hyperalgesia in a mouse model of chronic constriction injury (CCI)-induced neuropathic pain.

Methods: Intraperitoneal administration of Zer (5–50 mg/kg) from day 1 post-surgery was carried out to identify the onset and progression of the pain condition. Responses toward mechanical and cold allodynia, and mechanical and thermal hyperalgesia were assessed on days 3, 5, 7, 9, 11, and 14 post-surgery. Blood plasma and spinal cord levels of interleukin (IL)-1 β , IL-6, tumor necrosis factor- α , and IL-10 were screened using enzyme-linked immunosorbent assay on day 15.

Results: Zer (10 and 50 mg/kg) attenuated pain symptoms on all days of behavioral testing without any signs of sedation in the rotarod test. ED₅₀ values for mechanical allodynia, cold allodynia, thermal hyperalgesia, and mechanical hyperalgesia were 9.25, 9.507, 8.289, and 9.801 mg/kg, respectively. Blood plasma and spinal levels of IL-1 β , IL-6, and tumor necrosis factor- α but not IL-10 were significantly ($p < 0.05$) suppressed by zer treatment.

Discussion and conclusion: Zer exhibits its antiallodynic and antihyperalgesic properties via reduced sensitization at nociceptor neurons possibly through the suppression of inflammatory mediators. Zer may prove to be a novel and beneficial alternative for the management of neuropathic pain.

Keywords: zerumbone, allodynia, hyperalgesia, interleukin-1 β , interleukin 6, tumor necrosis factor- α

Introduction

Neuropathic pain is a highly prevalent global problem that affects almost 6.9%–10% of the population worldwide.¹ Approximately 40% of patients attending pain clinics for treatment of various forms of pain conditions exhibit neuropathic symptoms.^{2,3} Neuropathic pain arises due to factors that cause direct trauma to the nerve, tumour growth, cancer compression, and cancer therapy.⁴ Apart from autoimmune diseases and metabolic diseases such as diabetes, lifestyle of individuals such as smoking and alcoholism could also lead to this condition.^{5,6}

Neuropathic pain is defined as “pain that arises as a direct consequence of a lesion or diseases affecting the somatosensory system” and is often accompanied by symptoms

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of allodynia and hyperalgesia.⁷ This debilitating pain condition severely affects the physical activities of patients and further leads to emotional distress.⁸ As currently available conventional drugs are only partially effective, accompanied with undesired side effects, alternative pain management options are greatly sought-after.^{9,10}

The pathophysiology of neuropathic pain shows that inflammation is a vital component of the progression and maintenance of this condition.¹¹ When a nerve is injured, Wallerian degeneration takes place whereby axons and myelin sheaths undergo degradation. The vascular permeability is compromised, permitting infiltration of macrophages and various other immune cells such as mast cells to the injury site. Inflammatory cytokines, principally interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , and IL-6, are secreted by resident and non-resident macrophages.^{12,13} Inflammatory mediators are signaling proteins that take up a role as intercellular messengers as an immune response.¹⁴ They in turn recruit and activate macrophages, neutrophils, and surrounding glial cells at the site of injury.¹⁴ Primary afferent neurons are directly sensitized by TNF- α , IL-1 β , and IL-6 via their receptors.^{15–17}

Inflammatory cytokines have a primary role that triggers the downstream cascade of the inflammatory process, followed by the antiinflammatory process during the recovery and repair process. An “inflammatory soup” is formed comprising adenosine triphosphate, bradykinin, histamine, serotonin, prostaglandin E₂, leukotriene B₄, nerve growth factor, substance P, and calcitonin gene-related peptide contributing to neurogenic inflammation.¹⁸ Peripheral sensitization occurs, a result of which normally inactive and unresponsive “silent nociceptors” become sensitive to the development of spontaneous discharges, giving rise to allodynia.^{19,20} In addition, nociceptors, which usually transmit painful stimulus, are now hypersensitive and thus exhibit an exaggerated pain response, also known as hyperalgesia.¹⁹

Considering that neuropathic pain is largely modulated and maintained by the neuroimmune and neuroinflammatory activities, treatments or drugs often target to either reverse or reduce the inflammatory process.²¹ We have recently reported that zerumbone (Zer) (Figure 1), a monocyclic, sesquiterpenoid phytochemical substance isolated from the rhizome oil of *Zingiber zerumbet* (L.) Roscoe ex Smith (Zingiberaceae), was able to therapeutically attenuate symptoms of neuropathic pain.²² More recently, we have elucidated the involvement of the serotonergic and L-Arginine-Nitric Oxide-cGMP-K⁺ATP channel pathways in the analgesic action of Zer in neuropathic pain.^{23,24} This

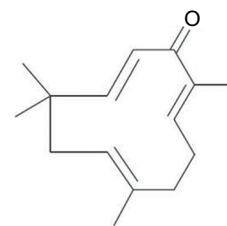


Figure 1 Chemical structure of zerumbone.

reinforces that Zer is a potential bioactive compound for the management of neuropathic pain.

Apart from its antinociceptive properties, Zer has also been widely reported for its antiinflammatory properties.^{25–27} Zerumbone successfully reversed the increase in IL-1, IL-6, and TNF- α production in the inflammatory response of diabetic rats.²⁸ Furthermore, zerumbone suppresses the synthesis of these inflammatory mediators in dextran sodium sulfate-induced colitis of mice.²⁹ Direct influence of zerumbone, specifically, on these aforementioned mediators has been well documented, not just in various other in vivo models but also in vitro.³⁰ However, suppression of the inflammatory process by zerumbone in a neuropathic pain model, specifically in the chronic constriction injury (CCI) model, is generally lacking and remains to be explored.

With this in mind, our study explored, for the first time, the prophylactic effects of Zer treatment administered from the initial stage throughout the progression stage of this neuropathic pain condition. We also provide evidence of the inflammatory components in the circulation as well as in spinal cord tissues following treatment with this compound. This approach with a prophylactic paradigm is expected to reverse the neuroimmune and neuroinflammatory processes. The significance of such treatments in clinical settings is that it serves as a preventive measure that could be started in advance for patients with higher risk of developing neuropathic pain, with the hope to delay or lessen the advancement of the condition.

Materials and methods

Zerumbone extraction and isolation

Zerumbone was prepared from rhizomes of *Zingiber zerumbet* using the same protocol as previously described by Perimal et al.²⁶ The plant was identified and confirmed by a botanist and a specimen was inserted at Institute of Bioscience, Universiti Putra Malaysia (Reference number: SK622/07). Briefly, rhizomes of *Zingiber zerumbet* were purchased from Chow Kit wet market, washed and sliced

into small pieces of ~0.5–1 mm in size. The hydrodistillation process was carried out by placing the rhizome slices in the round bottom flask with water added to it. Soluble oils were collected in a vial 4 h after water and hexane were added to it. After the separation of the hexane and water, the water phase was removed. The crude essential oil was obtained from the hexane phase using a rotary evaporator before being placed at 4°C for 48 h. Only the pure crystallized product of the compound was subjected to column chromatography. The yield was 0.07%. The purity was determined by subjecting the eluate to thin-layer chromatography. This was followed by recrystallization before the compound was analyzed with high-performance liquid chromatography (HPLC) where the purity was determined to be more than 98%. Zerumbone was dissolved in Tween-20, dimethyl sulfoxide (DMSO), and physiological saline 0.9% at a ratio of 5:5:90 and was prepared freshly just before the experiments.

Animals

Male ICR mice (25–35 g at the beginning of the experiments) were housed eight mice per cage under a 12 h dark/light cycle at a room temperature of 24°C±2°C and a humidity level of 70%, with free access to water and standard laboratory chow. Mice were acclimatized for 1 week prior to any experimental procedures. All animal experiments were performed in accordance to the ethical guidelines for investigation of experimental pain in conscious animals. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (Reference number: UPM/IACUC/AUP-R060/2013).

Chemicals

Tween-20, DMSO, amitriptyline, and morphine sulfate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Morphine was dissolved in 0.9% normal saline. All treatments were administered intraperitoneally (ip) at a volume of 10 mL/kg.

Preselection of mice and body weights

Mice were preselected with rotarod (UgoBasile, Varese, Italy) spinning at 20 rpm and only mice that were able to remain on the wheels for 120 s without falling or rolling over were used in the experiment to eliminate possible occurrence of motor dysfunction. Mice with approximately similar body weights were preselected to be used in the experiment and the weights were measured daily throughout the experimental period to monitor the effect of treatments on their feeding habits and general well-being.

Experimental design

A total of 56 mice were allocated into 7 groups (n=8 mice per group) as follows:

1. Sham
2. CCI + vehicle
3. CCI + Zer (5 mg/kg ip)
4. CCI + Zer (10 mg/kg ip)
5. CCI + Zer (50 mg/kg ip)
6. CCI + morphine (1 mg/kg ip)
7. CCI + amitriptyline (20 mg/kg ip)

Administration of drugs started 24 h post-surgery according to their respective groups and dosages. Drugs were given once a day for 14 days as described by Guneli et al.⁴²

CCI surgery

The procedure of Bennett and Xie³¹ was adapted with minor modifications to induce neuropathy.^{32,33} Briefly, mice were anesthetized with tribromoethanol (250 mg/kg ip) before an incision was made to the skin to expose the left common sciatic nerve. Three loose ligations with 1 mm spacing between ligatures were made using 4/0 silk sutures. A brief twitch in the left hind limb marks the tightness of the ligatures. The skin incision was closed using a non-absorbable suture, before iodine was applied to the area. The entire surgery procedure was carried out on mice in the sham group but with no ligations made to the sciatic nerve to confirm the consequence of nerve injury.

Nociceptive assay

Responses toward mechanical allodynia, cold allodynia, thermal hyperalgesia, and mechanical hyperalgesia were evaluated using von Frey, cold plate, Hargreaves, and Randall–Selitto tests, respectively. Baseline measurements of the nociceptive assays were obtained 1 day before CCI surgery (pre-CCI). Nociceptive assays were carried out 30 min following drug administration on days 3, 5, 7, 9, 11, and 14. The assays were carried out in a sequence of testing for mechanical allodynia, cold allodynia, thermal hyperalgesia, and mechanical hyperalgesia whereby this sequence was consistent throughout the experiment.

von Frey filament test

Response toward mechanical allodynia was evaluated by von Frey filament test as described by Chaplan et al³⁴ and Campana and Rimondini.³⁵ Mice were placed in a Plexiglass chamber on an elevated wire mesh grid and were allowed to acclimate prior to testing. When all explorative and grooming

behavior was reduced and the animal is on its four limbs, von Frey filament (IITC Life Science Inc., Woodland Hills, CA, USA) was applied to the middle of the dorsum of the ipsilateral paw followed by the contralateral paw. The mean of three readings was recorded as the withdrawal threshold.

Cold plate test

Response toward cold allodynia was evaluated by cold plate test as described by Nadal et al.³⁶ Mice were placed onto a cold plate (UgoBasile, Varese, Italy) at a temperature of $5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$. The number of times the mouse lifted, scratched, or flinched its paws were counted as the number of paw lifts. Each mouse was observed for a total of 5 min and measurements were obtained from both the ipsilateral and contralateral paws. The total number of lifts of the ipsilateral paw was then subtracted from the total number of lifts of the contralateral paws.

Hargreaves test

Response toward thermal hyperalgesia was assessed using Hargreaves test as described by Hargreaves et al³⁷ using Hargreaves apparatus (Model 37370, Gemonio, Varese, Italy). Mice were placed in a Plexiglass chamber which was placed on an elevated clear platform. Following a brief acclimatization period, a radiant heat source with an infrared radiation intensity of 80 W/sr was directed to the mid-plantar surface of its ipsilateral paw followed by the contralateral paw. The time taken for the mouse to either remove or flinch its paw was recorded as the withdrawal latency. The radiant heat source was removed after 20 s to prevent injury to the paws.

Randall–Selitto

Mice response toward mechanical hyperalgesia was carried out as previously described by Randall and Selitto.³⁸ Briefly, mice were restrained as such where they were able to flex their legs. A constantly increasing pressure was applied to the plantar surface of both the ipsilateral and contralateral hind paws through the tip of the paw pressure meter (IITC Life Science Inc., Woodland Hills, CA, USA). Readings were obtained when the mice attempted to withdraw the paw. The cut-off force was set at 200 g to prevent further injury to the paws.

Determination of ED_{50} values

The effective dose (ED_{50}) was determined for each nociceptive assay using GraphPad Prism V.5. The doses were $\log[\text{dose}]$ transformed and plotted against % of inhibition. A nonlinear regression (curve fit) was generated to determine the ED_{50} values.

Motor coordination

Possible sedative effects of the drug treatments were evaluated by the rotarod test (UgoBasile, Varese, Italy) as previously described by Zulazmi et al.²⁴ On day 14 post-CCI, mice were administered with their respective treatments. Exactly 30 min later, the animals were placed on the rotarod bar rotating at 20 rpm. The time spent on the bar for a period of up to 180 s was recorded. This method was carried out only in sham-operated and CCI mice treated with vehicle, Zer (50 mg/kg), morphine (1 mg/kg), and amitriptyline (20 mg/kg).

Euthanasia and organ collection

On day 15 post-CCI, treatments were given accordingly and mice were sacrificed 30 min posttreatment. Blood was withdrawn via cardiac puncture, centrifuged at 3000 g at 4°C and frozen at -20°C until assayed. The spinal cord at the lumbar 4–6 region was dissected out and homogenized using a Polytron device (Mississauga, Ont, Canada). Approximately, 750 μL PBS per 25 mg tissue was used to homogenize the tissues before samples were centrifuged for 10 min at 4°C at 12,000 g. The aliquot of the supernatant fraction was stored at -20°C until further use.

Enzyme-linked immunosorbent assay

Blood plasma and spinal cord cytokines (TNF- α , IL-1 β , IL-10, and IL-6) levels were measured using mouse-specific, enzyme-linked immunosorbent assay (ELISA) kits purchased from eBioscience, Vienna, Austria. Assays were performed according to the manufacturer's instructions. The samples were assayed in duplicates and the cytokines levels were expressed in pg/mg whereby 50 μL of blood plasma or tissue protein was used for each assay. Standards were run in every trial, and standard curves were generated for every assay. Sample concentrations were calculated using Bio-Plex Manager software.

Statistical analysis

All data obtained were checked for normality using Shapiro–Wilk Test for Normality before statistical testing was carried out. Data for behavioral testing were expressed as mean \pm SEM and were analyzed using two-way ANOVA followed by Bonferroni's post hoc analysis. Values for ELISA were expressed relative to the total protein. A confidence interval of 5% or less was considered to be statistically significant. All behavioral tests and ELISA assays were carried out where the investigator was blinded from the treatment group of the animals.

Results

von Frey filament test

Figure 2 represents the mean body weights of mice throughout the experiment. Mice with similar ($F(6, 49)=3.966, p=0.003$) at the beginning of the experiment showed no significant ($p>0.05$) changes between experimental groups on all days. Figure 3 represents the pain response toward mechanical allodynia when tested with von Frey filaments. Mice that underwent CCI surgery show significantly increased sensitivity as early as day 3 ($F(6, 49)=14.121, p=0.000$) and were significantly different ($p\leq 0.05$) from sham group throughout the experimental period. Treatment with Zer at a dose of 5 mg/kg did not show significant ($p>0.05$) difference from the vehicle group, indicating the lack of effect of this treatment dose on mechanical allodynia. However, Zer administered at higher doses of 10 and 50 mg/kg ip significantly attenuated mechanical allodynia as the values were significantly different from the vehicle group on all days of testing. Both morphine, 1 mg/kg ip, and amitriptyline, 20 mg/kg ip, that serve as positive controls, completely alleviated mechanical allodynia and showed mean values similar ($p>0.05$) to that of the sham group. The withdrawal threshold mean values of the contralateral paws were not affected by either the surgery or the treatments as the values were not significantly different from the sham group ($F(6, 49)=1.479, p=0.209$).

Cold plate test

Figure 4 shows the response cold allodynia which was tested using the cold plate test. All mice did not show any paw lifting in the baseline data (0.00 ± 0.00) ($F(6, 49)=0.000$;

$p=0.00$). Following CCI and treatment with vehicle, the mean number of paw lifting was recorded to be 16.29 ± 0.71 on day 14. Again, a significant ($p<0.05$) effect was observed only in Zer 10 and 50 mg/kg ip with mean values 6.43 ± 0.90 and 2.86 ± 0.53 , respectively. Even though both positive control drugs alleviated ($p<0.05$) cold allodynia, amitriptyline, 20 mg/kg ip was more effective with a mean value of 0.29 ± 0.18 on day 14. Cold allodynia was not observed in the contralateral paws.

Hargreaves test

The CCI model produced thermal hyperalgesia as significant increased pain response was observed in CCI groups compared to the sham group ($F(6, 49)=6.128, p=0.000$) as shown in Figure 5. Zerumbone at doses of 10 and 50 mg/kg ip alleviated thermal hyperalgesia on all days of behavioral testing. Both these doses showed similar ($p>0.05$) effect to morphine on days 9, 11, and 14. Amitriptyline completely reversed thermal hyperalgesia as the values were not significantly different ($p>0.05$) from the sham group. Thermal hyperalgesia was absent in the contralateral paws.

Randall–Selitto test

CCI surgery successfully developed mechanical hyperalgesia (Figure 6) as all groups subjected to CCI showed reduced threshold compared to sham-operated mice. Response toward mechanical hyperalgesia was significantly lowered ($F(6, 49)=55.731; p=0.000$) in higher doses of Zer (10 and 50 mg/kg), morphine (1 mg/kg ip), and amitriptyline (20 mg/kg ip) treated groups indicating the antihyperalgesic property of Zer and the positive control drugs. Lower dose

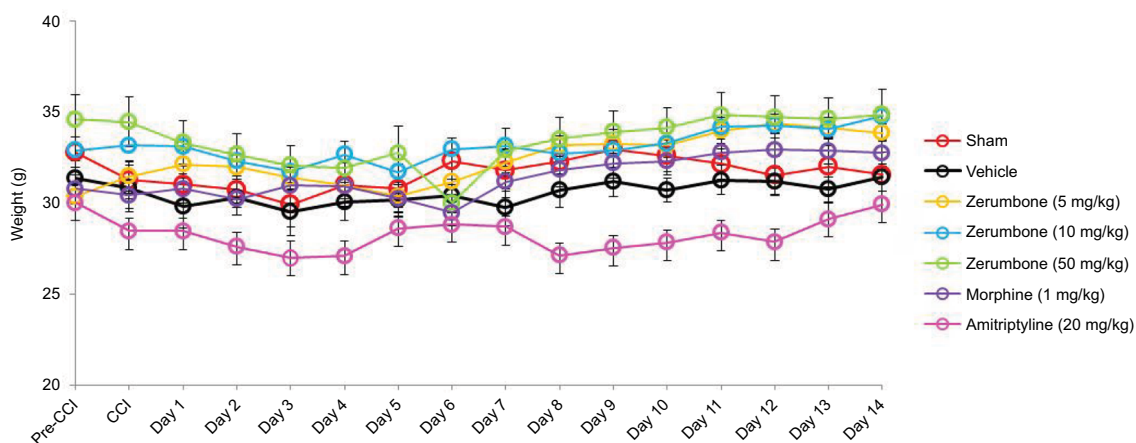


Figure 2 Changes in the body weights of mice in different treatment groups throughout the experimental period of 14 days.

Notes: Data are expressed as mean \pm SEM; $n=8$ mice per group, assessed by two-way ANOVA followed by Bonferroni's post hoc analysis. Filled circles indicate data significantly different ($p\leq 0.05$) from vehicle-treated group.

Abbreviations: ANOVA, analysis of variance; CCI, chronic constriction injury.

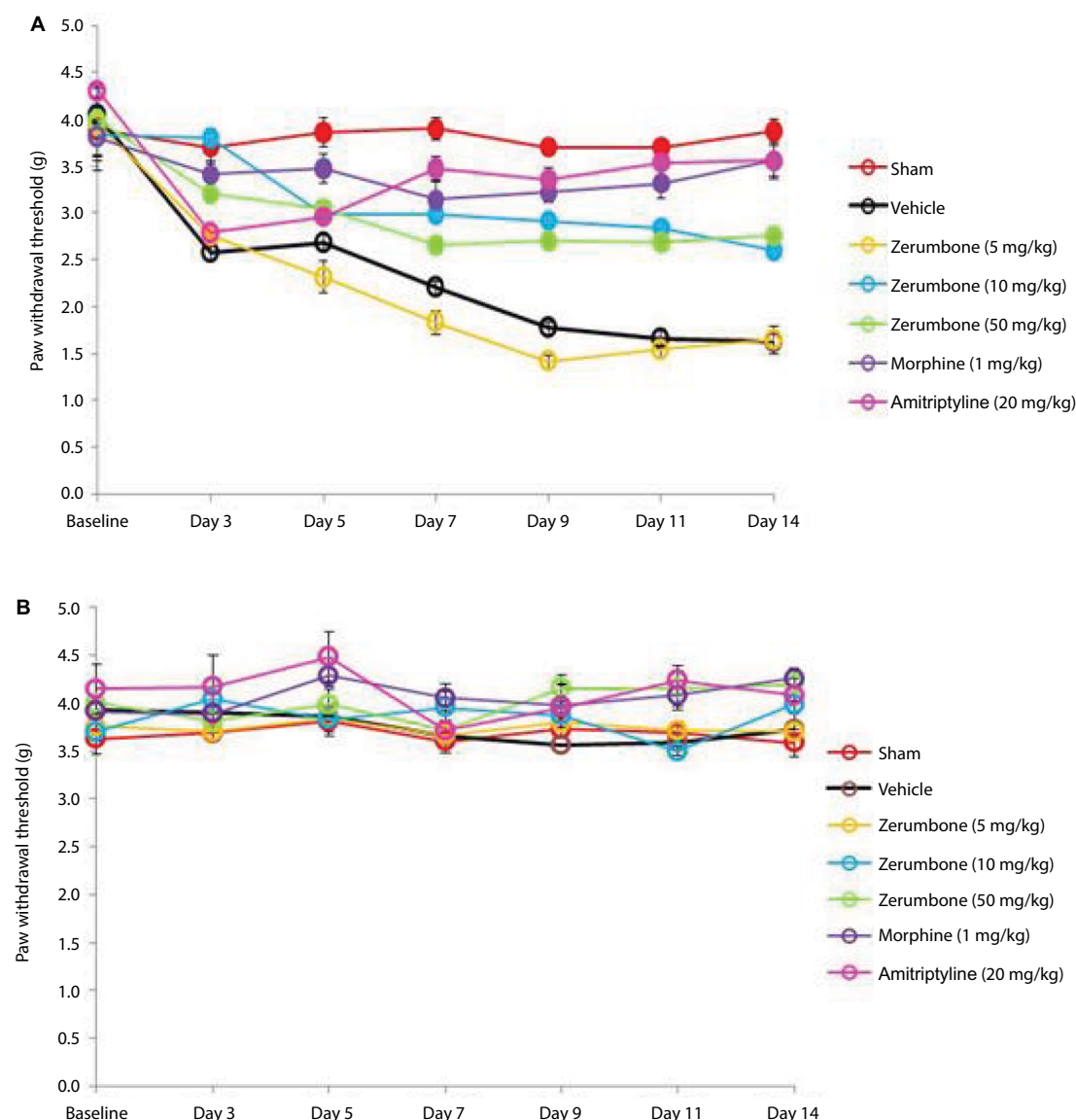


Figure 3 Effect of treatments on the (A) ipsilateral and (B) contralateral paw withdrawal thresholds toward mechanical allodynia as tested with von Frey filament test. **Notes:** Data are expressed as mean \pm SEM; $n=8$ mice per group, assessed by two-way ANOVA followed by Bonferroni's post hoc analysis. Filled circles indicate data significantly different ($p \leq 0.05$) from vehicle-treated group. **Abbreviation:** ANOVA, analysis of variance.

of Zer (5 mg/kg), however, failed to exhibit antihyperalgesic effect as the threshold values were not significantly different ($p > 0.05$) from the vehicle group. On the contralateral paw, mechanical hyperalgesia was absent and was not affected by the treatments.

Motor coordination test

Rotarod test was used to evaluate the presence of sedative effects of the treatments (Figure 7). Neither vehicle nor the highest dose of Zer (50 mg/kg ip), morphine (1 mg/kg ip), or amitriptyline (20 mg/kg ip) showed reduced effect, as all

mice were able to complete the entire rotarod session, which lasted for 3 min.

Enzyme-linked immunosorbent assay

Blood plasma

Figure 8 shows the circulating levels of inflammatory mediators in the CCI model and after the treatment of Zer (10 and 50 mg/kg). CCI caused an increase in pro-inflammatory cytokines, precisely IL-1 β , TNF- α , and IL-6. Zerumbone treatment successfully reduced IL-1 β , TNF- α , and IL-6 compared to the vehicle group. The levels IL- β and TNF- α

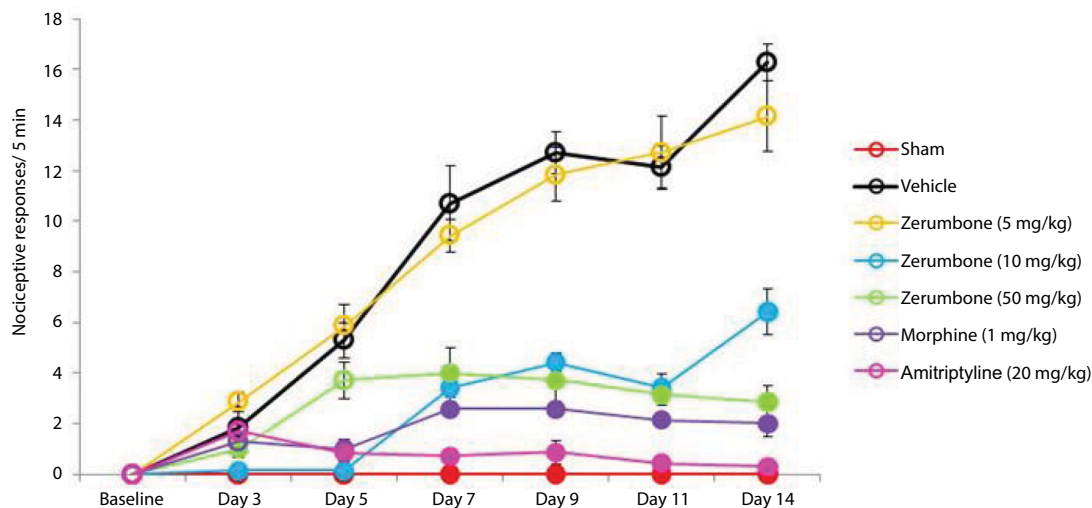


Figure 4 Effect of treatments on cold allodynia withdrawal response as tested with cold plate.

Notes: Data are expressed as mean \pm SEM; $n=8$ mice per group, assessed by two-way ANOVA followed by Bonferroni's post hoc analysis. Filled circles indicate data significantly different ($p \leq 0.05$) from vehicle-treated group.

Abbreviation: ANOVA, analysis of variance.

were reduced to similar levels ($p > 0.05$) of the sham group but IL-6 level was significantly higher ($p \leq 0.05$) than the sham group. The differences in the blood level of inflammatory mediators were not significantly different in both doses of Zer (10 and 50 mg/kg). However, blood plasma IL-10 showed no significant difference ($p > 0.05$) in all the groups tested, indicating that neither the CCI surgery nor Zer treatments altered the levels.

Lumbar region of the spinal cord

Figure 9 represents the levels of inflammatory mediators in the lumbar 4–6 of spinal cord following CCI and treatment with Zer (10 and 50 mg/kg). The levels showed similar trend with the blood levels of inflammatory mediators where IL-1 β , TNF- α , and IL-6 were reduced ($p \leq 0.05$) with Zer treatment but no changes ($p > 0.05$) in IL-10 level was detected. Interleukin-6 level was significantly higher ($p \leq 0.05$) than its sham counterpart. Higher dose of zerumbone (50 mg/kg) was not able to exhibit a significant difference ($p > 0.05$) from the lower dose of Zer (10 mg/kg) used in the assays.

Discussion

This study aimed to highlight the effects of prophylactic treatment of Zer at three different doses on attenuation of allodynia and hyperalgesia as well as the changes in production of inflammatory mediators. The outcome showed that all the drugs used as treatment did not cause any form of discomfort, change in appetite, or inability to access food

throughout the period of 14 days as the body weights did not undergo any drastic changes. Hyperalgesia and allodynia were observed as early as our first day of behavioral testing on day 3 possibly due to the axon degeneration process that occurs from 24 to 48 h in mice.³⁹ Zerumbone successfully exerted analgesia at doses of 10 and 50 mg/kg but not 5 mg/kg throughout the experimental period. This outcome is consistent with our earlier study by Zulazmi et al.²² This indicates that Zer treatment, both therapeutically and prophylactically, resulted in similar outcomes.

Morphine, the “gold standard” drug for pain treatment, and amitriptyline, a first-line drug for neuropathic pain treatment, were used as positive controls in this study. Even though these drugs were very effective in the attenuation of allodynia and hyperalgesia, reversing the threshold/latency similar to that of the sham group, Zer (10 and 50 mg/kg) was comparable with these drugs at some points. Most importantly, the effects of all the drugs were validated to be behavior specific since mice receiving the highest dose of drugs were able to maintain their balance on the rotarod throughout the 180 s time period. This ruled out the possibilities that the reduced pain response is due to suppression of general behavior as a result of the sedative effects of the drugs.⁴⁰ The ED₅₀ values of Zer were calculated to be 9.245 mg/kg ($R^2=0.9894$), 9.507 mg/kg ($R^2=0.9794$), 8.289 mg/kg ($R^2=0.9105$), and 9.801 mg/kg ($R^2=0.9157$) for mechanical allodynia, cold allodynia, thermal hyperalgesia, and mechanical hyperalgesia, respectively. Therefore, the best dose for prophylactic Zer treatment is 10 mg/kg.

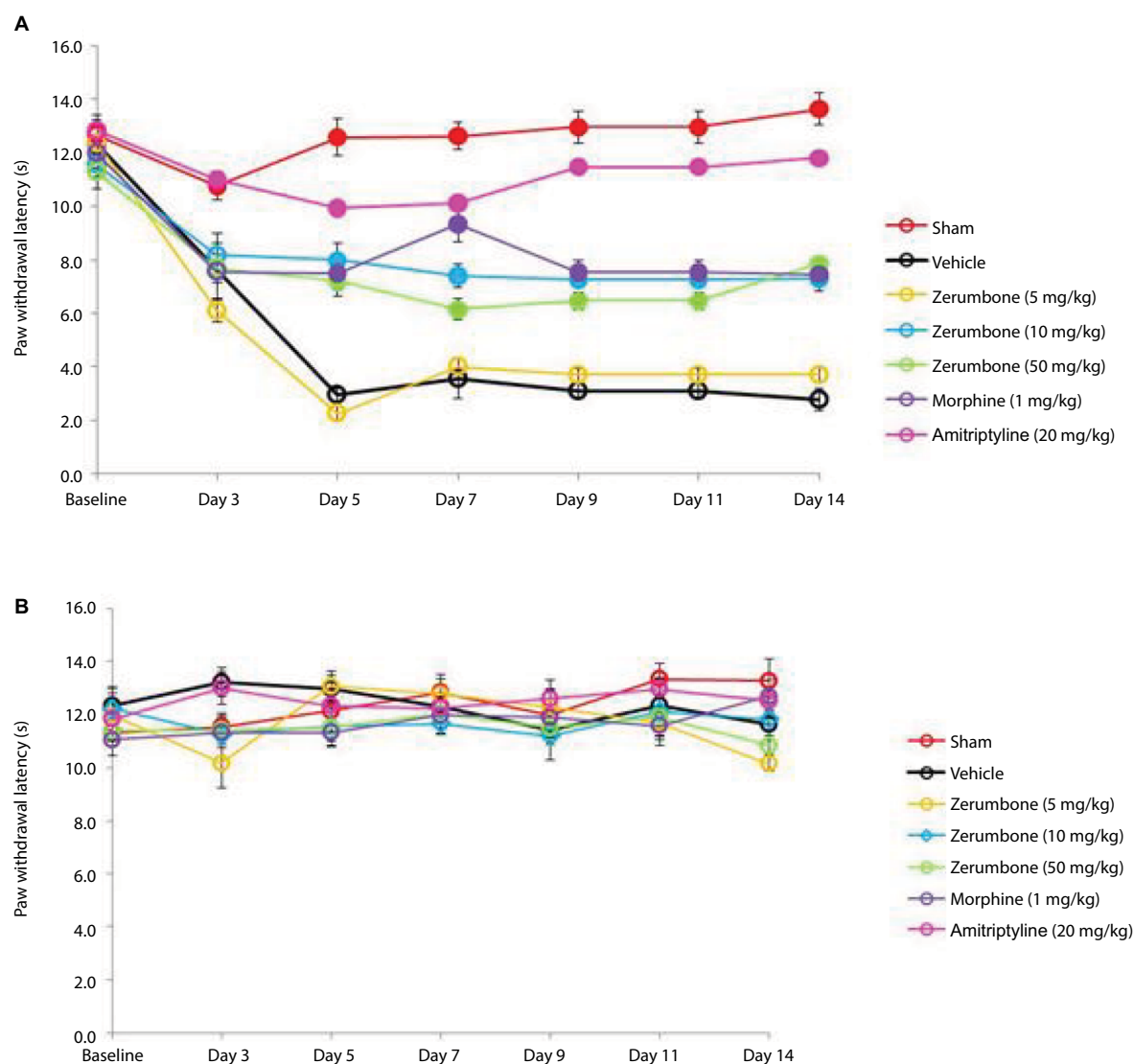


Figure 5 Effect of treatments on the (A) ipsilateral and (B) contralateral paw withdrawal latency toward thermal hyperalgesia as tested with Hargreaves.

Notes: Data are expressed as mean \pm SEM; $n=8$ mice per group, assessed by two-way ANOVA followed by Bonferroni's post hoc analysis. Filled circles indicate data significantly different ($p \leq 0.05$) from vehicle-treated group.

Abbreviation: ANOVA, analysis of variance.

We carried out this study on the CCI model of neuropathic pain as it involves both components of nerve injury and inflammation.⁴¹ The results showed that the CCI model induced upregulation of plasma and spinal cord IL-1 β , TNF- α , and IL-6 observed in the vehicle group on day 14, in line with previous studies.^{41–43} IL-1, TNF- α , and IL-6 are expressed as early as the second or third day post injury, during the first phase of Wallerian degeneration process. IL-1 β reached maximum levels after 24 h of nerve injury and again showed peak production on day 14.⁴⁴ Within hours of nerve injury, mRNA coding of TNF- α and TNF receptors were rapidly increased in the sciatic nerve and subsequently

within 1–3 days in the dorsal root ganglion (DRG), with the elevated levels displayed for at least 2 weeks post-injury.^{45,46}

The action of Zer in pain modulation could take place at different levels of the pain pathway which include nerve terminals, DRG, spinal cord, and the ascending or descending tracts to the supraspinal regions. Furthermore, Zer could also act on various ion channels that are present along the peripheral nerves. In this study, Zer treatment suppresses inflammatory mediators, lowering pain transmission from primary afferent neurons to the ascending tract of the pain pathway. Pain impulses reaching the supraspinal regions will be modulated, and thus lower pain levels are perceived.

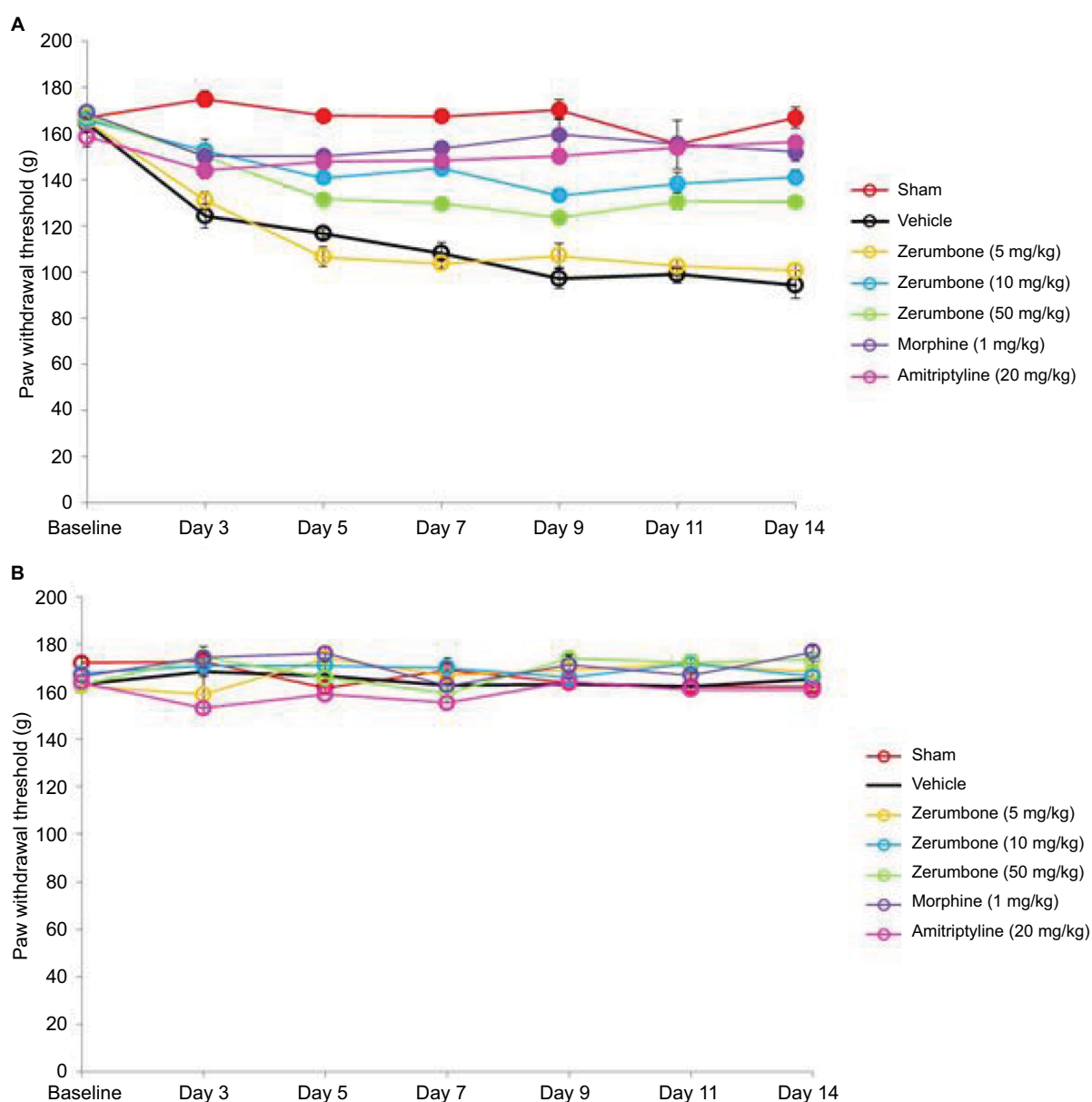


Figure 6 Effect of treatments on the (A) ipsilateral and (B) contralateral paw withdrawal threshold toward mechanical hyperalgesia as tested with Randall–Selitto in CCI and sham mice.

Notes: Data are expressed as mean \pm SEM; $n=8$ mice per group, assessed by two-way ANOVA followed by Bonferroni's post hoc analysis. Filled circles indicate data significantly different ($p \leq 0.05$) from vehicle-treated group.

Abbreviations: ANOVA, analysis of variance; CCI, chronic constriction injury.

This is then translated as lowered pain response that we have observed in our behavioral study.

The role of IL-6 in the pathogenesis of neuropathic pain has also been reported. In a sciatic cryoneurolysis (SCN) rat model where nerve injury is induced by freezing the sciatic nerve, elevated IL-6-like immunoreactivity and IL-6 mRNA were observed in the spinal cord dorsal and ventral horns of the rats.^{47,48} Furthermore, administration of human IL-6 intrathecally could also mimic and cause pain behavior after SCN.⁴⁷ The levels of IL-1 β and TNF- α are also elevated in

the DRG and spinal cord following peripheral nerve injury. Glial cells, principally astrocytes and microglia that are present within the dorsal horn, are activated to play essential roles in pain transmission and maintenance of this chronic disease.^{12,49} These glial cells secrete IL-1 β , IL-6, and TNF- α which are characterized as messengers of the immune system to act on various cells, including neurons.⁴⁹

Since pro-inflammatory cytokines are critical in the initiation and maintenance of neuropathic pain, antagonism of these cytokines' signaling inhibits neuronal hypersensitivity

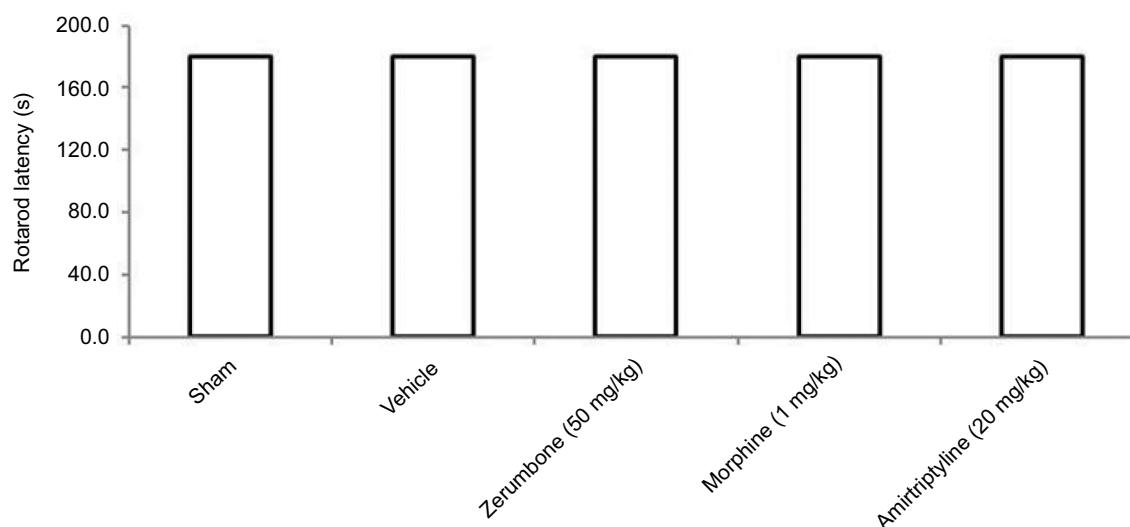


Figure 7 Effect of the treatments on the rotarod performance in CCI and sham mice.

Notes: Data are mean \pm SEM; $n=8$ mice per group.

Abbreviation: CCI, chronic constriction injury.

and inflammation due to nerve injury. TNF- α inhibitors have been reported to reduce peripheral nerve injury-associated thermal and mechanical pain.⁵⁰ Receptor fusion protein, etanercept, and humanized monoclonal antibody, infliximab, are examples of TNF- α inhibitors. Etanercept effectively reduced pain in clinical trials when tested in patients suffering from sciatica and spinal stenosis.^{51,52} Infliximab, on the other hand, benefited patients with L3–L4 and L4–L5 disc herniation.⁵³ Furthermore, TNF- α inhibitors are currently used to treat a number of immune disorders including psoriasis, Crohn's disease, and rheumatoid arthritis.⁵⁴

IL-1 β alleviates thermal and mechanical pain hypersensitivity following spinal nerve injury and CCI in mice.^{55,56} Therapeutic targets that are currently approved and used in clinical settings include anakinra and rilonacept, which target IL-1 receptors. Canakinumab is an anti-IL-1 β neutralizing antibody used in a few inflammatory diseases such as gout, rheumatoid arthritis, and Still's disease.⁵⁴

IL-6 inhibitors have also been established as therapeutic targets. Tocilizumab, a humanized IL-6 receptor neutralizing antibody, could be used in the treatment of juvenile idiopathic arthritis, rheumatoid arthritis, and sciatica.⁵⁷ Intrathecal injection of anti-IL-6 antibody reduced mechanical allodynia induced by L5 spinal nerve transection.⁴⁸ Furthermore, attenuation and delay of mechanical allodynia were observed in IL-6 knockout mice following sciatic nerve ligation and CCI.^{58,59}

Zerumbone's antiinflammatory properties by suppression of these biomarkers have been extensively reflected across

various models. Zerumbone administered intravenously successfully downregulates IL-1 β expression and attenuates the severity of acute necrotizing pancreatitis induced by sodium taurocholate as well as pancreatitis-induced hepatic injury.⁶⁰ On the other hand, expression of TNF- α was inhibited by intraperitoneal injection of Zer in acute and chronic models of inflammation in mice.²⁷ The secretion of serum IL-6 and membrane-bound IL-6 receptors was inhibited by Zer in human cervical cancer and human ovarian cancer cell lines.⁶¹

Furthermore, oral doses of Zer were able to inhibit TNF- α expression and cytosolic IL-6 in the ultraviolet B photokeratitis and cataractogenesis models.^{62,63} In addition, Zer significantly lowered IL-1 β and TNF- α in the colonic mucosa of a dextran sodium sulfate-induced acute ulcerative colitis model.²⁹ The p38 MAPK activation of IL-1 β , IL-6, and TNF- α was decreased by treatment with Zer in a streptozotocin-induced diabetic nephropathy model in rats.⁶⁴ These studies indicate that Zer is involved in the regulation of all these three inflammatory cytokines.

In this study, treatment of Zer from the first day of injury suppressed production of pro-inflammatory mediators as the levels of both the circularity and spinal cord IL-1 β , TNF- α , and IL-6 were lowered. Upregulated levels of inflammatory cytokines (TNF- α and IL-1 β) trigger the activation and expression of phospholipase-A₂ (PLA₂), an enzyme essential for the initial degradation of myelin sheath after nerve injury. Since the release of these cytokines was suppressed in Zer-treated mice, it was expected

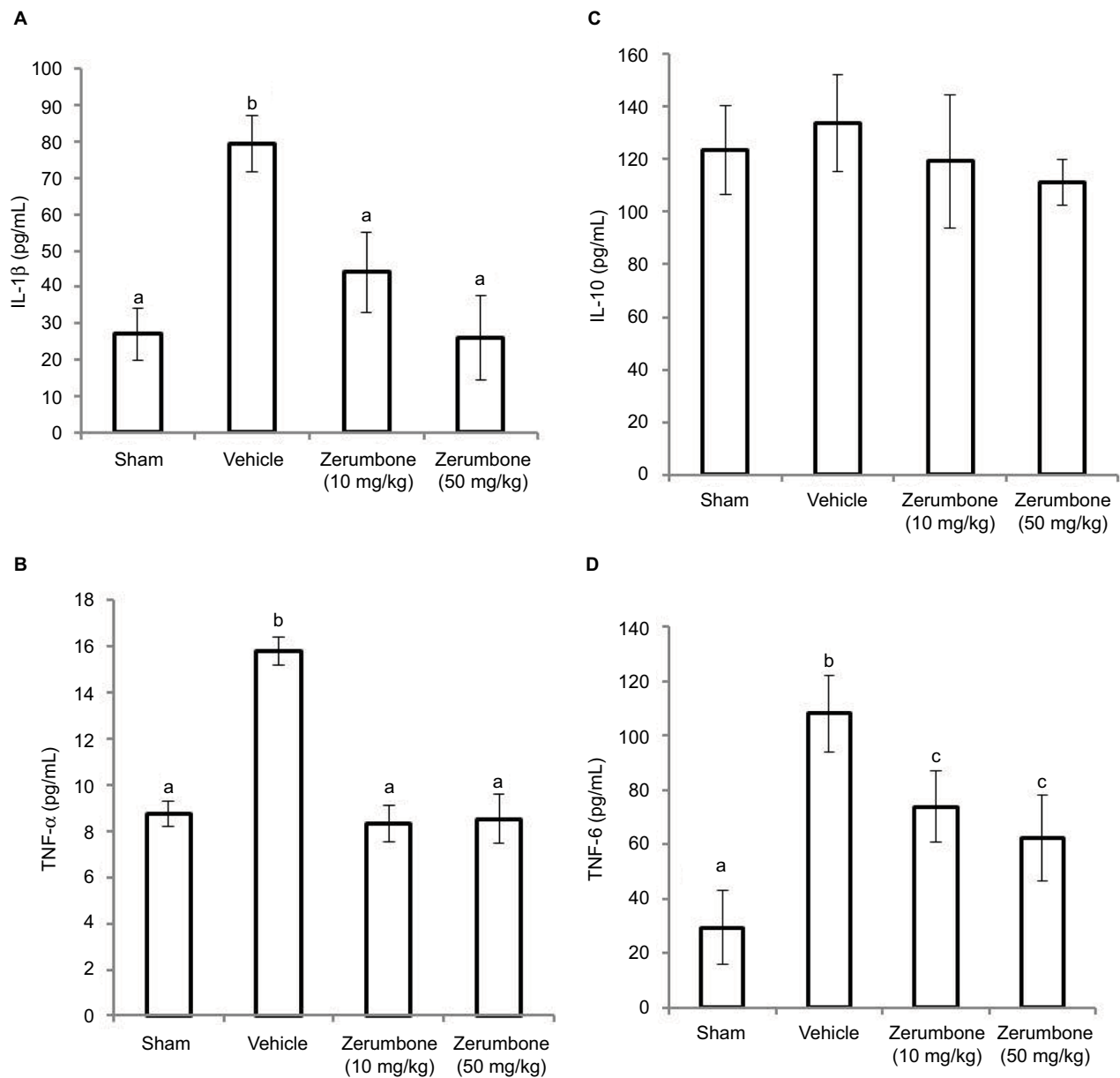


Figure 8 Effect of zerumbone (10 and 50 mg/kg) treatments on the blood plasma levels of (A) IL-1β, (B) TNF-α, (C) IL-10, and (D) IL-6 in CCI and sham mice.

Notes: Values represent mean ± SEM; n=6 mice per group. ^{a,b,c}Columns with different superscripts are significantly different at $p \leq 0.05$.

Abbreviations: CCI, chronic constriction injury; IL-1β, interleukin-1beta; TNF-α, tumor necrosis factor-alpha.

to stop or at least reduce myelin break down in the sciatic nerve.

The antiinflammatory cytokine, IL-10, however, assumes a role in attenuating the inflammatory process by counter-regulating the pro-inflammatory mediator productions and functions while upregulating endogenous anti-cytokines.⁶⁵ IL-10 is usually expressed after the recruitment of macrophages.¹³ Apart from that, IL-10 also plays a protecting role against tissue damage and excessive immune responses. The immune homeostasis is usually maintained by the release of endogenous antiinflammatory cytokines, specifically IL-10,

which follows a negative feedback mechanism by suppressing the genes that code pro-inflammatory cytokines and their respective receptors. In addition, IL-10 also has analgesic actions. By evaluating the changes in the production of this cytokine in this study, it would allow clearer understanding of the association of Zer with the inflammatory process that takes place within.⁶⁶

IL-10 was indifferent in this CCI model and Zer-treated groups on day 14, contrary to earlier reports of peak production of this mediator in the first 2 weeks after injury.⁶⁵ However, there are also reports stating that the expression

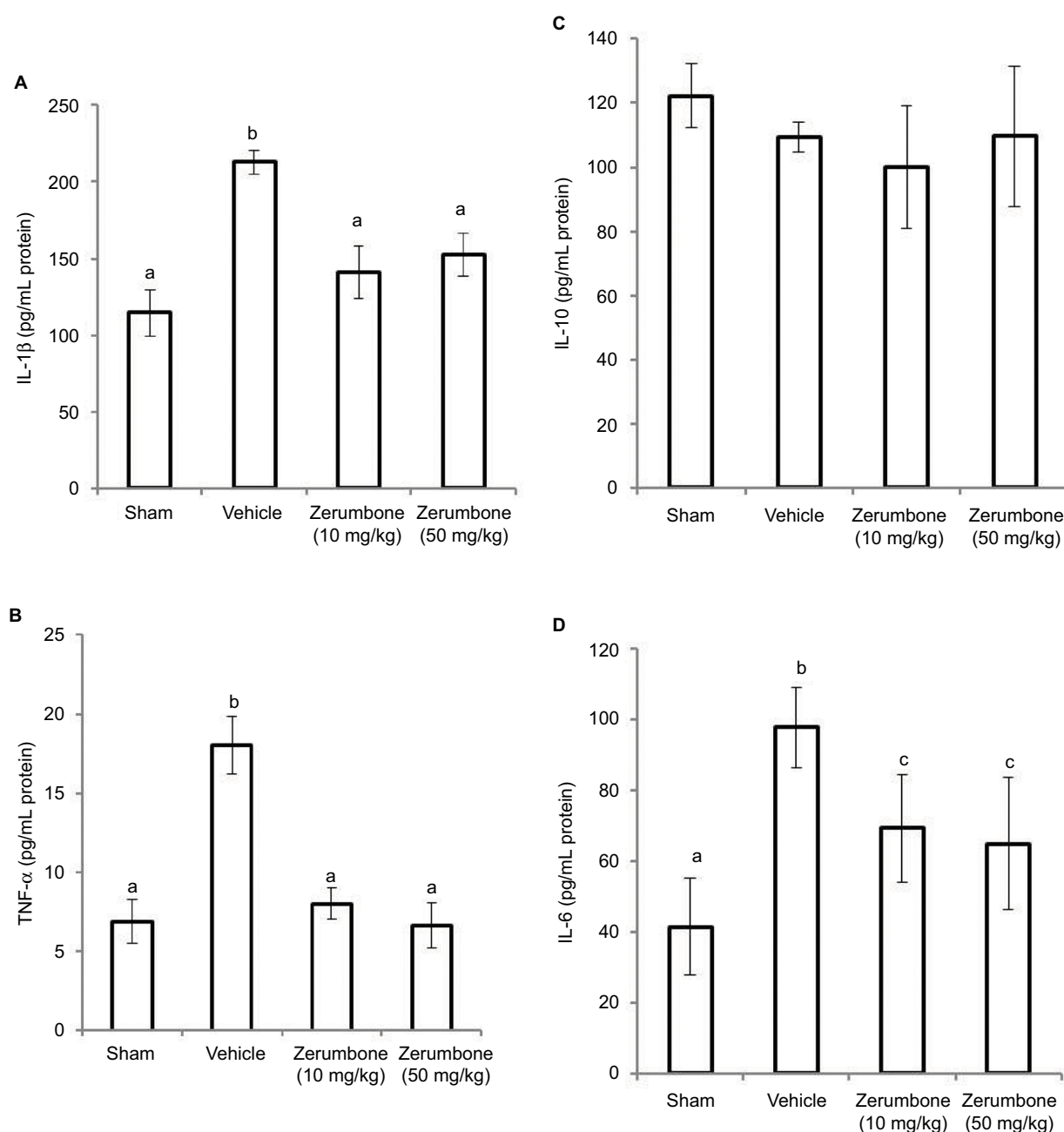


Figure 9 Effect of zerumbone (10 and 50 mg/kg) treatment on the L4–L6 spinal cord levels of (A) IL-1β, (B) TNF-α, (C) IL-10, and (D) IL-6 in CCI and sham mice.

Notes: Values represent mean \pm SEM; n=6 mice per group. ^{a,b,c}Columns with different superscripts are significantly different at $p \leq 0.05$.

Abbreviations: CCI, chronic constriction injury; IL-1β, interleukin-1beta; TNF-α, tumor necrosis factor-alpha.

of this mediator is more gradual and progressive whereby a significant mRNA expression is reached only at day 45 post-injury.⁶⁷

Besides these principal cytokines evaluated in this study, other key components of inflammation such as prostaglandin E2 (PGE₂), nuclear factor (NF)-κB, nitric oxide (NO), and inducible nitric oxide synthase (iNOS) are also involved in peripheral nerve injury. Zerumbone's antiinflammatory effect in many other components of inflammation has been reported

in various other models in both in vivo and in vitro.^{27–29,60,62,68–75}

Nevertheless, this study is the first report on the antiinflammatory role of Zer in the CCI mouse model of neuropathic pain. Figure 10 represents an overview of Zer's antiinflammatory properties in attenuating allodynia and hyperalgesia.

Conclusion

In conclusion, Zer was consistent in exhibiting its analgesic properties toward mechanical allodynia, cold allodynia,

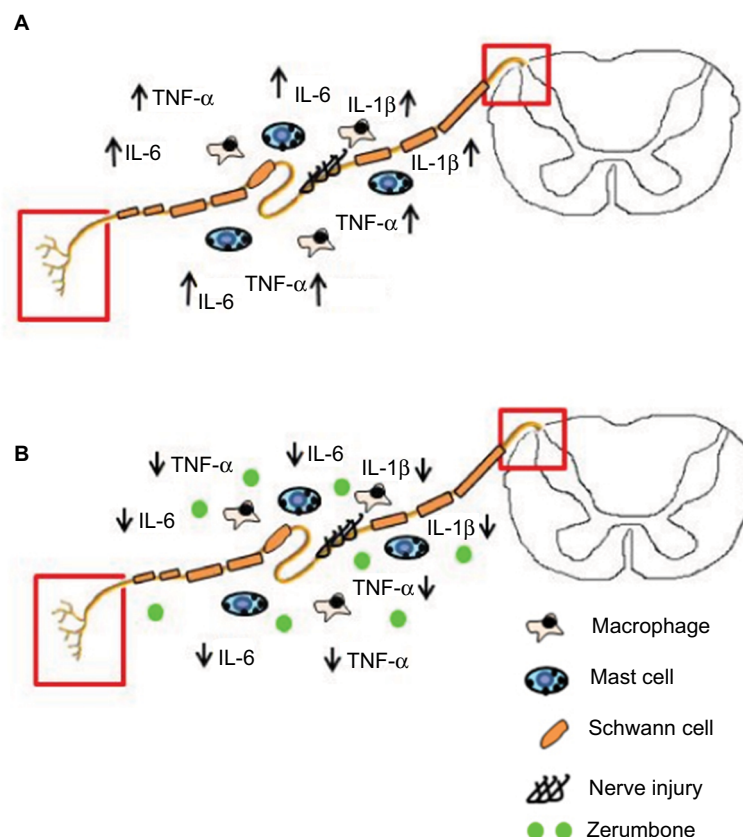


Figure 10 Overview of the anti-inflammatory actions of zerumbone on neuropathic pain symptoms allodynia and hyperalgesia.

Notes: (A) In an event of nerve injury, resident and blood-borne macrophages and mast cells rush to the site of injury. These cells release inflammatory mediators such as IL-1 β , TNF- α , and IL-6. These mediators cause heightened sensitivity at nociceptor terminals and dorsal horn of spinal cord (red box) leading to behavioral symptoms such as allodynia and hyperalgesia. (B) Treatment of zerumbone lowers the levels of inflammatory mediators (IL-1 β , TNF- α , and IL-6) in the blood and spinal cord. This prevents hypersensitivity at nociceptor terminals and dorsal horn of spinal cord (red box) resulting in the attenuation of allodynia and hyperalgesia.

Abbreviations: IL, interleukin; IL-1 β , interleukin-1 beta; TNF- α , tumor necrosis factor-alpha.

mechanical hyperalgesia, and thermal hyperalgesia when Zer treatment was provided throughout the development and progression of neuropathic pain. This outcome is due to the anti-inflammatory property exerted by Zer. We demonstrated that Zer successfully reversed the production of IL-1 β , IL-6, and TNF- α but not IL-10 in blood plasma as well as in spinal cord tissues. Therefore, Zer is a highly potential drug for the management of pain in neuropathic conditions when administered prophylactically through suppressing the inflammatory event that occurs due to nerve injury.

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Authors' contribution

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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